A prospective study on the surgical treatment of morbid obesity: effects of weight loss on obesity related comorbidities and chronic inflammation

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Voor Claudia en Julie

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Chapter 1

General introduction

Introduction

Defining the problem

Obesity is becoming a serious problem in the western society. It is a lifelong, progressive disease of fat storage, manifested by medical, physical, psychological, social and economic problems, of which the presentation is related to the amount of excess weight[1-3]. The number of overweight and obese Northern Americans has continued to increase since 1960, a trend that is not slowing down (Figure 1.1). Today, 65.7 percent of adult Northern Americans and already 16.5% of children are classified as being overweight (body mass index (BMI)>25 kg/m^2) or obese, 30.6% obese (BMI>30 kg/m^2) and 5.1% morbidly obese (BMI>40 kg/m^2)[4].

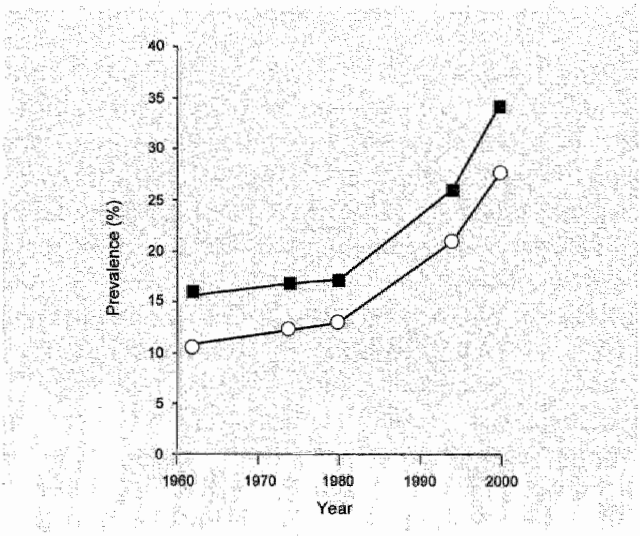

Figure 1.1 Between 1960 and 2000 prevalence of obesity (BMI≥30 kg/m^2) increased tremendously in adults in the USA, both for men (circles) and female (black boxes).

The rise in prevalence of obesity is associated with an increase in obesity related comorbidities (e.g., type 2 diabetes, hyperlipidemia, hypertension, obstructive sleep apnoea, heart disease, stroke, asthma, back and lower extremity weight bearing degenerative problems, several forms of cancer, depression etc.)[5-7]. These comorbidities are responsible for more than 2.5 million deaths per year worldwide[5] and at least 300,000 in the United States alone, competing with smoking as a public health threat[8,9]. The loss of life expectancy due to obesity is profound. In comparison with a normal-weight individual, a 25-year–old morbidly obese man has a 22% reduction in expected

remaining lifespan, representing an approximate loss of 12 years of life[10]. The comorbidities also lead to an enormous amount of extra medical costs. In 2003 alone, the medical costs in the USA, directly attributable to obesity, were a staggering US$ 75 billion ![11]

This trend of increasing prevalence of obesity is also present in the Netherlands. Approximately 12% of the adult Dutch population is already obese and 1.5% is morbidly obese, and this prevalence has roughly doubled over the past 20 years[12]. Unfortunately, patients with morbid obesity are usually unable to control their body weight by conservative means. Therefore there is a need for an effective weight reducing treatment. A recent review demonstrates that professional conservative treatments, such as medical therapy with orlistat (an inhibitor of gastric and pancreatic lipases[13]), only slightly reduce body weight in obese subjects (mean weight loss was 3.3 kg in 2 years). Also low-fat diets (including 600 kcal/day deficit diets) resulted in a mean weight loss of only 5.3 kg, over a 3 years period. The addition of an exercise program or behavioural therapy was associated with improved weight loss and lowered risk factors for at least 1 year[14]. Whether the effect lasts longer than 1 year is not known yet. Next to this it is unclear whether both exercise and behavioural therapy together further enhanced the effect of diet[14]. As a result of these disappointing findings of conservative treatments, surgical therapy for morbid obesity (bariatric surgery) is becoming a more and more accepted therapy for patients with extreme overweight. Already in the late fifties pioneers in the surgical field started to develop treatments for morbidly obese subjects. The first bariatric procedure by Kremen et al. was performed in animals and published in 1954[15]. They presented a jejuno-ileal bypass (JIB), involving the joining of the upper small intestine to the lower part of the small intestine, bypassing a large segment of the small bowel. This leaves out a large part of the nutrient absorptive circuit, leading to extensive weight loss. It did not take many years before the first morbidly obese patients were operated with a JIB. Initially, the results were promising and patients lost a substantial amount of body weight. However, the many complications and complaints like uncontrollable diarrhoea, dehydration, liver disease in approximately 30%, electrolyte imbalance and others, were sufficiently distressing both to patient and doctor to cause the procedure to fall into disrepute. This led to a search for alternative procedures, like biliopancreatic diversion, Roux-en-Y gastric bypass, vertical banded gastroplasty and since approximately 1990 adjustable gastric banding. All these different procedures had one common goal: to reduce body weight in order to reduce the obesity related comorbidities (Figure 1.2).

Gastric restrictive surgery has been proven to effectively reduce excess weight in morbid obese patients. Furthermore it was demonstrated that weight loss after gastric restrictive surgery leads to a reversal, elimination or significant

amelioration of many obesity related diseases like diabetes, hyperlipidemia, hypertension, gastro-oesophageal reflux and obstructive sleep apnea[6,16-18]. With respect to type 2 diabetes, about 85% of patients with diabetes experience improvement in their diabetes course after bariatric surgery[19-21]. Furthermore, also the effect on hyperlipidemia is impressing. In many reports after surgery an approximately 10-fold lowering of the incidence compared to a control group was shown[22-26]. Hypertension resolved in 55-69% of the patients after surgery[21,27-29] and when correctly placed and adjusted appropriately laparoscopic banding procedures are also an effective treatment for gastro-oesophageal reflux disease[16,30-32].

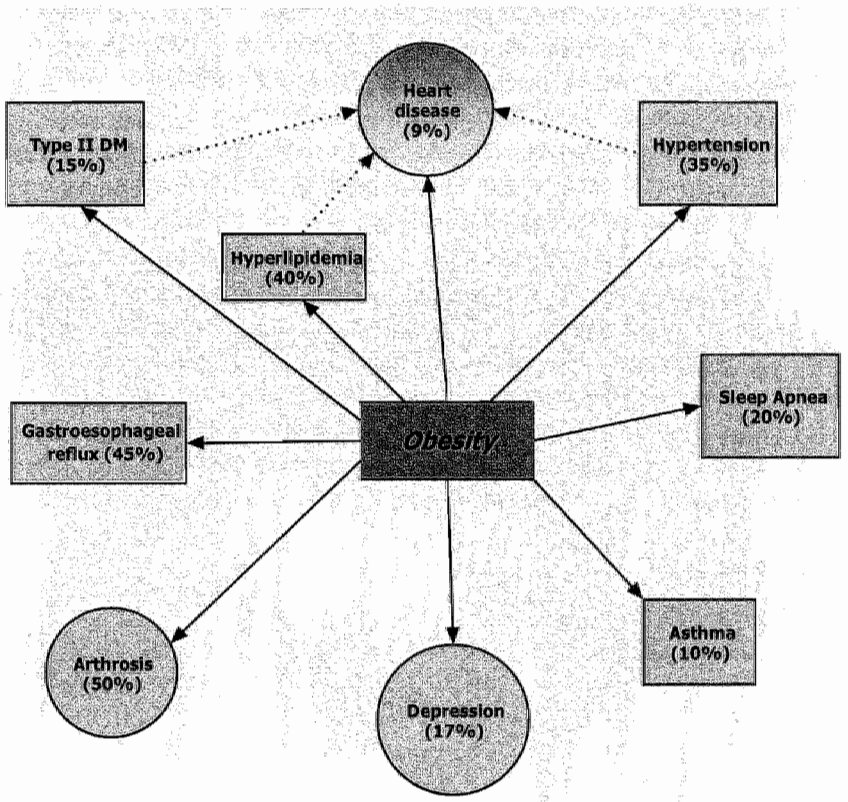

Figure 1.2 Obesity and related comorbidities.
The percentages of obese patients suffering from the specific comorbidity are retrieved from Buchwald et al.[6].

In addition, a growing amount of evidence relates increased successful gastric restrictive surgery with longevity. MacDonald et al. reported that obese diabetic

patients treated with an oral hypoglycaemic had a 4.5% annual mortality rate for every 9 years of follow-up compared with a 1% mortality rate in obese diabetic patients who underwent gastric bypass[33]. Furthermore, Christou et al. demonstrated that weight-loss surgery in 1035 patients compared with 5746 controls with a 5-year follow-up reduced the relative risk of death by 89%, with an absolute mortality reduction of 5.5%[34].

In our institute studies were undertaken to investigate the cost-effectiveness of bariatric surgery. In 1999 van Gemert et al. were the first to report on the potential benefit of surgical treatment of morbid obesity to society and demonstrated that treatment of morbid obesity with vertical banded gastroplasty, as described by Mason et al.[35], is cost-effective[36]. These findings were confirmed by Sampalis et al. who demonstrated that 3.5 years after bariatric surgery, the initial investment for the weight-reduction surgery and related hospital care was compensated by a reduction in total cost (Figure 1.3)[37]. In short, bariatric surgery is essential in order to control the increasing prevalence of morbid obesity and obesity related comorbidities and reduces the accompanied increased health care costs.

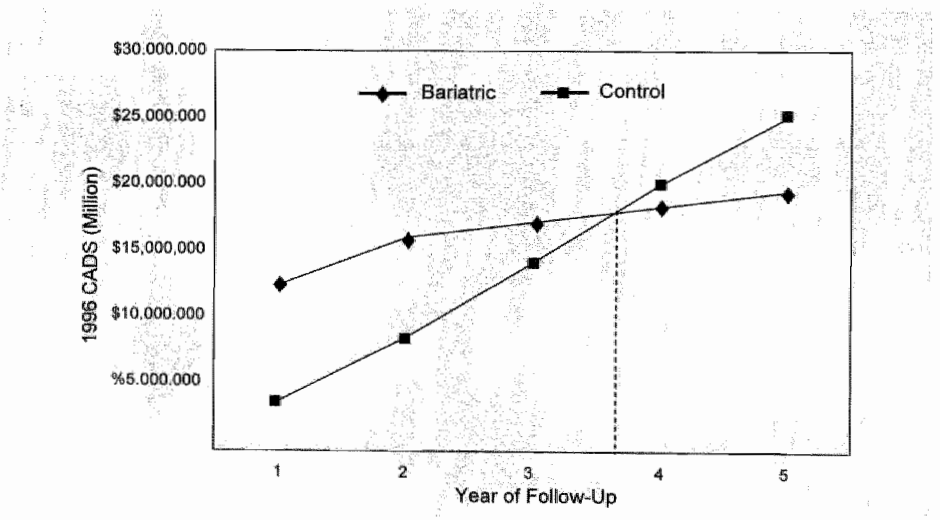

Figure 1.3 Influence of bariatric surgery on health-care costs.
Average cumulative costs per 1,000 patients per year for hospitalization after bariatric surgery (black squares) or control group (black boxes). Reproduced with permission from Obesity Surgery (ref. 37).

The vertical banded gastroplasty (VBG) is the standard surgical treatment for morbid obesity in the University Hospital Maastricht (Figure 1.4A). In this type of open gastric restrictive surgery, the stomach volume is reduced by making a

small pouch (<20 ml). Since 1993, another type of gastric restrictive surgery, the laparoscopic adjustable gastric banding (Lap-Band) operation (Figure 1.4B), has been performed in many countries. It was introduced in Maastricht in 1996. To evaluate the potential benefit of this new method, a prospective randomized trial was initiated and described in this thesis in which both types of gastric restrictive surgery were compared. Furthermore, the different comorbidities were studied. In particular, the effect of bariatric surgery on insulin resistance is studied in this thesis. Insulin resistance is one of the major obesity related comorbidities which has been shown to improve following weight reduction[38-41]. Insulin resistance is defined as the body's inability to respond to, and use, the insulin it produces. Hence subjects with insulin resistance are at risk for developing type 2 diabetes. However, the aetiology of insulin resistance in obesity and the mechanism that underlies the improvement of insulin resistance, as seen after weight reduction, is unclear. Many explanations are proposed for the aetiology of insulin resistance in obesity. Adipose tissue itself has been suggested to play an important role in the development of insulin resistance. This role of adipose tissue is emphasized by the adverse metabolic consequences of adipose tissue excess as well as adipose tissue deficiency. Adipose tissue excess (or obesity), is associated with the development of features of the so-called metabolic syndrome including insulin resistance[2,42-44]. Interestingly, adipose tissue deficiency or lipodystrophy also leads to insulin resistance, both in man and mice[45]. Moreover, transplantation of adipose tissue in lipodystropic mice leads to normalisation of the insulin sensitivity[46]. Thus, the development of insulin resistance in both excess and deficiency of adipose tissue justifies the search for a pathophysiological role of adipose tissue herein.

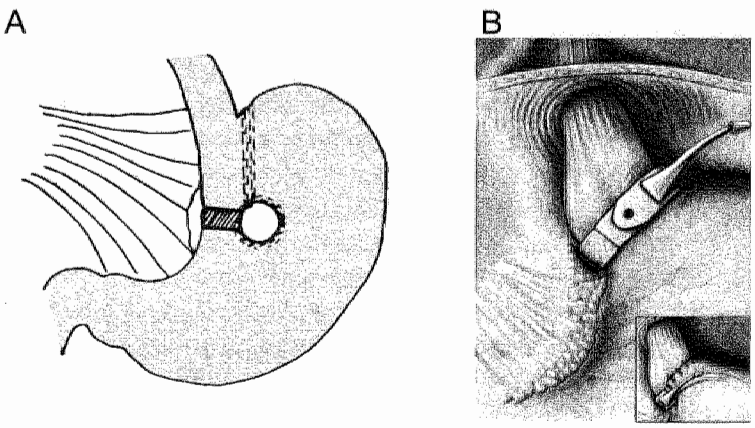

Figure 1.4 Vertical banded gastroplasty (A) and Lap-Band (B).

Both *in vitro* and *in vivo* studies provided considerable evidence for a causal role of inflammatory mediators in obesity induced insulin resistance and type 2 diabetes mellitus[47,48]. *In vitro*, the pro-inflammatory cytokine TNFα has been shown to affect insulin signalling[47]. This effect is mediated by activation of serine kinases that increase serine phosphorylation of insulin receptor substrate-1 and -2, making them poor substrates for insulin receptor kinases and increasing their degradation. TNFα also impairs insulin signalling indirectly by increasing serum nonesterified fatty acids (NEFAs), which have independently been shown to cause insulin resistance in multiple tissues[49].
An interesting clinical observation is the presence of marked insulin resistance in patients with elevated levels of circulating TNFα[50-56]. Moreover, insulin resistance is usually present in patients with sepsis and is directly proportional to the severity of the stress response[57]. During sepsis, insulin induced tyrosine phosphorylation of the insulin receptor substrate IRS-1 and subsequent activation of the p110-type phosphatidylinositol 3-kinase (PI 3-kinase) is impaired resulting in defective GLUT-4 receptor translocation, diminished glucose uptake, insulin resistance in skeletal muscle, and hepatic insulin resistance[58]. The mechanism whereby these alterations are induced in sepsis is unknown, but increased levels of TNFα are suggested to play a key role[59-65].
Whether inflammatory processes are also responsible for the development of obesity induced insulin resistance is unclear. An enhanced inflammatory state in morbidly obese patients as evidenced by increased plasma concentrations of cytokines and acute phase proteins without direct clinical evidence of acute or chronic inflammation was reported in these patients[66-69]. These data may suggest a causal relation between inflammation and insulin resistance in obese patients. Some recent crucial observations support the role of inflammation in obesity induced insulin resistance. The first observation regards mouse strains deficient for factors controlling inflammation which are resistant to obesity induced insulin resistance. Notably, mice lacking TNFα, one of the major inducers of inflammation, are resistant to obesity induced type 2 diabetes[70]. Furthermore, mice carrying a heterozygous deletion of I Kappa B Kinase 2 (IKK2), the most important kinase mediating NF-κB activation upon TNFα signalling, develop a resistance to diet induced or obesity induced insulin resistance[71]. These data are supported by Arkan et al. who demonstrated in IKK-β deficient mice, both in hepatocytes and myeloid cells, that insulin sensitivity remained present after a high fat diet[72].
Taken together strong evidence exists that the transcription factor NF-κB, a key player in inflammatory processes, is crucially involved in obesity induced insulin resistance.
A second target of TNFα signalling is c-Jun amino-terminal kinase (JNK). In the absence of JNK, mice also do not develop insulin resistance[73]. Further observations regard independent studies using genome wide expression

studies (micro-array analysis) on adipose tissue of murine models of obesity induced insulin resistance, but also in *in vitro* studies on human adipocytes. These studies led to the discovery that the expression of inflammatory mediators observed in adipose tissue can be attributed to the presence of large number of adipose tissue homing macrophages[74,75]. Subsequently it was hypothesized that adipose tissue homing macrophages are the mediators of obesity induced inflammation and insulin resistance. Besides adipose tissue homing macrophages also adipose tissue itself appears to be of importance for the production of TNFα and regulation of insulin sensitivity[76-78]. In a recent study of Furukawa et al. it was demonstrated that accumulation of fat leads to increased oxidative stress in adipose tissue which on its turn leads to the production of adipokines (adipose tissue derived cytokines), TNFα, Il-6 and PAI-1[79].

A macronutrient intake was shown to induce oxidative stress and inflammatory responses. Glucose and fat challenge induces oxidative stress, which was demonstrated to activate, on its turn, at least 2 major proinflammatory transcription factors, NF-κB and AP-1[80-83]. Because obesity is often accompanied by a relative increased food intake, this could be one of the mechanisms that underlie the proinflammatory state and development of insulin resistance in obese subjects.

Very recently a new pathway for the upregulation of inflammatory mediators was discovered. Özcan et al. demonstrated that obesity causes endoplasmic reticulum (ER) stress in peripheral tissues[84]. This leads to suppression of insulin receptor signalling through hyper activation of JNK and subsequent serine phosphorylation of insulin receptor substrate-1. The authors postulate that ER stress underlies the presence of the stress and inflammatory responses in obesity.

The adipokine leptin is potentially of importance in the upregulation of inflammatory mediators in obese individuals. Leptin, is a 16 kDa polypeptide containing 167 amino acids with structural homology to cytokines. Leptin is secreted in direct proportion to adipose tissue mass as well as in response to nutrition. The secretion is relatively greater from subcutaneous compared to visceral adipose tissue[85,86]. Interestingly however, in contrast to the ob/ob leptin deficient mice, in which exogenous leptin reduces obesity, neither endogenous high leptin levels nor treatment with exogenous leptin are effective in reducing obesity in man[87]. These data may suggest a state of leptin resistance in obese individuals of which the pathophysiological mechanism is unknown but may result from defects in leptin signalling or transport across the blood-brain barrier[88,89]. On the other hand, a functional state of leptin resistance could also be regulated via soluble leptin receptors which compete for leptin-cellular leptin receptor interactions[90]. In order to get more insight into these leptin-leptin

receptor interactions, soluble leptin receptor levels are studied in this thesis in morbidly obese and weight loosing subjects.
Although initially viewed as a satiety hormone, increasing evidence to date suggests that leptin influences the human energy balance through appetite but appears not to be involved in regulating energy expenditure[91]. However, other reports indicate a role for leptin in inflammatory processes. Leptin is able to induce upregulation of pro-inflammatory cytokines, both *in vivo* and *in vitro*[92]. Leptin modulates the T-cell immune response by increasing Th1- and suppressing Th2-cytokine production[93]. In contrast to leptin-deficient mice, which are obese due to the lack of leptin, in man obesity is associated with increased plasma leptin concentrations[94]. These elevated plasma leptin concentrations in morbidly obese patients could be responsible for the enhancement of constitutive immunological stimuli, leading to increased concentrations of acute phase proteins and other inflammatory markers, characteristic for the chronic inflammatory state in obese individuals. However, exogenous leptin in healthy, obese and obese diabetic subjects did not lead to an increase of inflammatory markers, indicating no etiopathogenic role for leptin in the inflammatory state often seen in obese individuals[95,96].
In short, at this stage the presence of enhanced levels of inflammatory markers in morbidly obese subjects is considered to be related with pathophysiological mechanisms that underlie the development of obesity related diseases and insulin resistance in particular. However, to which extend these inflammatory mediators are causative for the lower insulin sensitivity and how these inflammatory mediators are upregulated in obese individuals remains to be resolved.
After bariatric surgery, patients loose an extensive amount of body weight within a short period of time and are therefore a unique population for studying the effect of weight loss on different obesity related diseases.

The aim of this thesis is to compare the effects of two different types of gastric restrictive surgery in morbidly obese subjects on weight loss and postoperative complications. In addition, the effect of gastric restrictive surgery on gastric myoelectrical activity was studied. Furthermore, in this patient group underlying mechanisms of obesity related comorbidities were studied.
In this context the relation of weight and weight loss on inflammatory mediators was investigated. One of the most important obesity related comorbidity (insulin resistance) was studied in detail.

Outline of the thesis

The main object of the studies described in this thesis was to investigate the effects of gastric restrictive surgery on obesity related comorbidities. In addition we searched for insight in the relation between obesity, inflammation and insulin resistance.

In the first part of the thesis (Chapter 2) a prospective randomized trial of two different types of gastric restrictive surgical techniques Lap-Band vs. open VBG is described. Besides the effects on weight loss and comorbidities also complication rates were evaluated. Furthermore, the effect of extensive weight loss on gastro-intestinal motility disorders was evaluated (Chapter 3). Morbid obesity is often associated with gastro-oesophageal motility disorders like gastro-oesophageal reflux and dyspepsia. In order to evaluate the effect of gastric restrictive surgery on gastro-oesophageal motility, gastric myoelectrical activity, an indirect measure for gastric motility, was assessed before and after surgery.

Earlier studies found evidence for an association between high body weight and elevated plasma levels of inflammatory mediators. In this context we studied the correlations between BMI and plasma levels of different inflammatory mediators in a group of subjects with a BMI ranging from 20 to 61 kg/m^2 (Chapter 4) and the effect of extensive weight loss after gastric restrictive surgery on these inflammatory mediators (Chapter 5).

In Chapter 6 the effect of weight loss following bariatric surgery on circulating leptin and soluble leptin receptor levels was evaluated. In this context, techniques to measure the soluble leptin receptor levels and methods to elucidate the interaction between leptin and its soluble receptor were developed.

In the last experimental chapter the effect of extensive weight loss after gastric restrictive surgery on insulin resistance and its relation with inflammatory mediators was studied (Chapter 7). It is shown by others that insulin resistance improves after gastric restrictive surgery. In this context, we studied the effect of weight loss in morbidly obese subjects after gastric restrictive surgery on insulin resistance and inflammatory mediators and their mutual relation.

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Chapter 2

Laparoscopic adjustable gastric banding versus open vertical banded gastroplasty. A prospective randomized trial

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Abstract

Background

Laparoscopic adjustable gastric banding (LAGB) and open vertical banded gastroplasty (VBG) are treatment modalities for morbid obesity. However, few prospective randomized clinical trials (RCT) have been performed to compare both operations.

Methods

100 patients (50 per group) were included in the study. Postoperative outcomes included hospital length of stay (LOS), complications, percent excess weight loss (%EWL), BMI and reduction in total comorbidities. Follow-up in all patients was 2 years.

Results

LOS was significantly shorter in the LAGB group. Three LAGB were converted to open (1 to gastric bypass). Directly after VBG, 3 patients needed relaparotomies due to leakage, of which one (2%) died. After 2 years, 100% follow-up was achieved. BMI and %EWL were significantly decreased in both groups but significantly more in the VBG group compared to the LAGB group (31.0 kg/m^2 and 70.1% vs. 34.6 and 54.9% respectively). Comorbidities significantly decreased in both groups in time. 2 years after LAGB, 20 patients needed reoperation for pouch dilation/slippage (n=12), band leakage (n=2), band erosion (n=2) and access-port problems (n=4). In the VBG group, 18 patients needed revisional surgery due to staple-line disruption (n=15), narrow outlet (n=2) or insufficient weight loss (n=1). Furthermore, eight VBG patients developed an incisional hernia.

Conclusions

This RCT demonstrates that, despite the initial better weight loss in the VBG group, based on complication rates and clinical outcome, LAGB is preferred. It had a shorter LOS and less postoperative morbidity.

Introduction

Morbid obesity (body mass index (BMI) >40 kg/m^2) is a major problem in the developed and developing world[1,2]. The serious physical, psychological and economic comorbidities of obesity progress with the increase in excess weight. Gastric restrictive surgery has been proven to reduce the excess weight and thereby decrease the risk of life-threatening diseases, such as type II diabetes and coronary heart disease.

In our hospital, the standard surgical treatment for morbid obesity was the vertical banded gastroplasty (VBG) as described by Mason et al.[3]. In this type of open gastric restrictive surgery, the stomach volume was reduced by making a tiny pouch (<20 ml). Since 1993, a new type of gastric restrictive surgery, laparoscopic adjustable gastric banding (LAGB), has been performed in many countries. In this type of operation, an adjustable gastric band is placed laparoscopically around the very proximal stomach, which allows control of the outlet diameter and enables weight loss due to diminished food intake. The postoperative weight loss following LAGB appears to be more gradual than with most other surgical procedures[4-6]. Until now, only a few prospective studies have been performed and only recently have randomized trials been published assessing the long-term effectiveness of LAGB compared to VBG[7-10]. At introduction of the LAGB in our hospital in 1996, it was decided to compare the two restrictive operations.

Therefore, a prospective randomized clinical study was initiated to compare both gastric restrictive operations in morbidly obese patients.

Materials and methods

Subjects and study design

From May 1999 until December 2001, 100 morbidly obese patients were included in the study for a follow-up period of 2 years. Patients were considered eligible for the study if their BMI was >40 kg/m^2 or >35 kg/m^2 with comorbidities. All patients were between 18 and 60 years of age and had failed previous non-surgical attempts at weight loss. Patients who had previous obesity surgery or gastric surgery as well as patients with severe psychological disorders were excluded from the study.

After obtaining informed consent, patients were randomly assigned to LAGB or open VBG using a computer-generated randomization list, made before the start of the study. Patients were informed about their surgical treatment group during admission to the hospital. Demographic data, BMI, medication, medical history and comorbidities such as joint problems (primarily arthritic),

cardiovascular problems, pulmonary disorders, diabetes, hypertension, etc. were recorded. Comorbidities were recorded when patients were medically treated for the disorder. Hospital length of stay (LOS) and intra- and postoperative complications were recorded. The patients were admitted the day before surgery to the Surgical Department of University Hospital Maastricht. LAGB patients were generally sent home the day after the operation, resulting in a minimal hospital stay of 3 days. After VBG, patients were generally sent home on the 3rd day after surgery, resulting in a minimal stay of 5 days. After discharge, all patients were evaluated in the outpatient clinic at 3, 6, 12 and 24 months postoperatively.
The study was approved by the ethical committee of the University Hospital Maastricht.

Surgical technique

Anesthetic method and technique were similar for both groups. All patients were given a single dose of preoperative antibiotic.
VBG was performed as described by Mason[3]. In short, a midline incision was made from xyphoid to a point above umbilicus. A tiny gastric pouch of approximately 15-30 ml was created with a 4-row linear stapler (TA-90B, US Surgical Corp., Norwalk, CT) precisely to the angle of His. A Dacron band 5.0 cm in circumference, placed through the window formed by a circular stapler (Premium Plus CEEA 31 mm, US Surgical Corp., Norwalk, CT), left a very small stoma for food to pass from the small pouch to the remaining stomach. Because of the limited capacity of the gastric pouch, the amount of ingested food before onset of satiety was considerably limited leading to extensive weight loss[11].
LAGB (Lap-Band, INAMED, Santa Barbara, CA) was performed through five abdominal trocars. Intra-abdominal pressure was maintained at 14 mmHg. The band was placed around the uppermost part of the stomach. For definitive positioning of the band, a 15 ml calibration balloon was advanced by the anesthetist, placed below the cardia, and pulled up to the gastroesophageal junction. The band was closed under this pouch, and three or four sutures were placed anteriorly below and above the band, to ensure stable position.
All patients were extubated and transferred to the surgical ward postoperatively, unless they required ventilatory support or close observation in the intensive care unit. A nasogastric tube was not used routinely in the postoperative period.
Because of a reduced operating-room capacity, inclusion of all patients took 2.5 years instead of the planned one year. In total, 32 patients were operated according to the classical "peri-gastric" procedure described by Belachew et

al.[12] and the last 18 patients were operated using the improved method, the "pars flaccida" technique[13].
During the study period, the LAGB was insufflated when weight loss was insufficient (approximately <1 kg per week), with the first insufflation at least 6 weeks postoperatively. Adjustments of the LAGB where done frequently and only with small amounts (0.25-0.5 ml saline) except for the first adjustment (2 ml). The LAGB was adjusted 3-5 times within the first 2 years postoperatively.

Statistical analysis

Data are given as mean ± standard deviation. Statistical analysis was performed non-parametrically and two-sided. The Wilcoxon signed rank test was used to analyze differences between preoperative and postoperative values, within the morbidly obese subjects. Furthermore, the Mann-Whitney U test was used to analyze the differences in postoperative complications between both groups as well as between the peri-gastric and pars flaccida techniques in the LAGB group.
Statistical analyses were performed using the SPSS 10.0.7 statistical package. $P<0.05$ was denoted as statistically significant.

Results

Preoperative patient demographics

As shown in Table 2.1, 100 patients were included in the study: 50 patients underwent an open VBG and 50 patients underwent a LAGB of which 1 patient was converted during operation to a gastric bypass due to a perforation during retro-gastric tunnelling. BMI, age and sex did not differ between the groups. Mean total number of comorbidities was comparable in both groups.

Table 2.1 Patient demographic preoperative data.

	VBG (n=50)	LAGB (n=50)	*P*-value
Age	39 ± 8.5	37.2 ± 9.7	NS[a]
Sex (male / female)	10 / 40	10 / 40	NS
BMI (kg/m^2)	46.6 ± 6.4	46.7 ± 6.1	NS
Total comorbidities	1.3 ± 1.1	1.3 ± 1.0	NS

[a] Not significant

Hospital stay and early postoperative complications

In this study, 100% follow-up in both groups was achieved. LOS in the VBG group was significantly longer than LOS in the LAGB group (6.8±10.4 days (range 2-56) vs. 3.5±1.5 (range 2-9) respectively, $P<0.001$). The reason for this difference appears to be the strong difference in early postoperative complications. VBG operated patients had more postoperative complications during the hospital stay compared to LAGB operated patients (Table 2.2).

Table 2.2 Immediate postoperative complications for VBG and LAGB.

Complications	Number (percentage)	Mortality
VBG-group (n=50)		
Leakage, for which re-operation	3 (6%)	
Splenectomy (peroperative)	2 (4%)	
Obstruction (gastroscopy)	2 (4%)	
Infections	5 (10%)	
Sepsis	3 (6%)	1 (2%)
Urinary tract infection	1 (2%)	
Pneumonia	3 (6%)	1 (2%)
Wound infection	1 (2%)	
LAGB-group (n=50)		
Conversion to open LAGB	2 (4%)	
Conversion to Gastric Bypass	1 (2%)	
Infections	0	
Mortality	0	

In the VBG group, a total of 9 patients had perioperative complications. During VBG, in two patients a splenectomy was performed due to iatrogenic injury. Three patients had signs of leakage, for which re-operation was necessary within the first postoperative week. One of these patients died due to sepsis. This was the only patient with 5 comorbidities preoperatively. One patient died due to a pre-existing pneumonia, which was not reported to the surgeon preoperatively; immediately after VBG this patient developed high fever and pulmonary insufficiency from which he did not recover. In total, 7 infectious events were recorded in 5 patients: 3 had sepsis, 3 pneumonia and 1 urinary infection. Furthermore, 2 VBG patients developed temporary outlet obstruction early postoperatively due to a foreign body for which gastroscopy was necessary. However, these patients eventually had an uneventful recovery.
In the LAGB group, much less peri-operative complications were observed. Two patients were converted during operation to an open procedure due to technical problems. One patient was converted to gastric bypass due to perforation during retro-gastric tunnelling. No other peri-operative complications were observed; there were no infections and no peri-operative deaths.

Weight loss and long term complications

All patients reported had completed 2 years of follow-up. BMI significantly decreased in both groups (Figure 2.1). At 2 years, BMI decreased in the VBG group from 46.4±6.4 preoperatively, 31.1±6.2 at 1 year, to 31.0±6.0 kg/m^2 at 2 years postoperatively. In the LAGB group, BMI decreased from 46.7±6.1, via 35.0±6.3, to 34.6±6.5 kg/m^2 at 2 years. This decrease in BMI in time was significantly more in the VBG group compared to the LAGB group ($P \leq 0.002$).
In line with BMI, percentage of excess weight loss (%EWL), as determined by the 1983 Metropolitan Life Insurance Tables[14], increased in the VBG group to 71.1±24.0 and 70.1±25.5% at 1 and 2 years after operation respectively. In the LAGB group, %EWL increased to 53.3±21.2 at 1 year and 54.9±23.3% at 2 years after operation. This increase in EWL was significantly different at both time-points between the VBG and LAGB groups ($P \leq 0.001$), in favour of the VBG group.

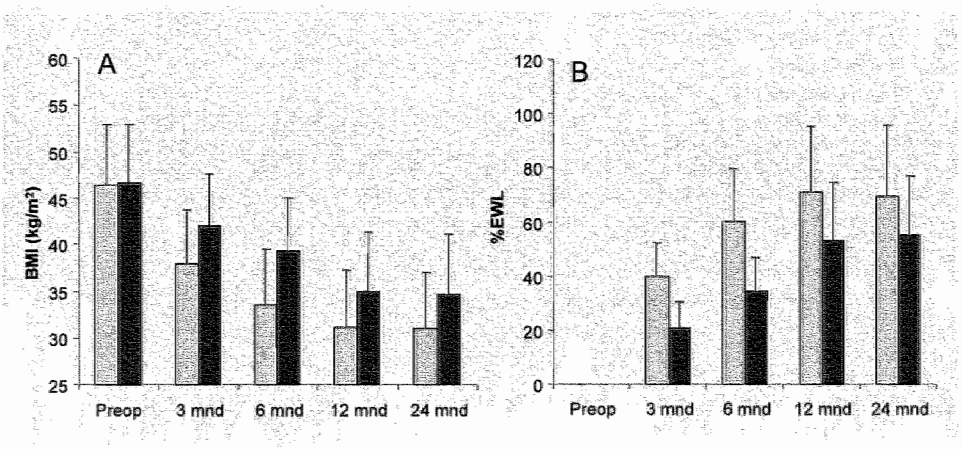

Figure 2.1 BMI (A) and Excess weight loss (B) after LAGB (dark gray bars) and open VBG (gray bars).

The long-term complication-rate was also assessed (Table 2.3). In the VBG group, 18 patients needed revisional surgery. In 15 patients, re-operation was necessary due to staple-line disruption with subsequent weight regain. In two patients, a too narrow outlet with vomiting and food intolerance was the indication for re-operation, and in one patient, insufficient weight loss without staple-line disruption was the indication. In addition to these 18 patients, another 8 patients in the VBG group developed an incisional hernia for which surgical repair was indicated. Furthermore, gastroscopy was needed one or

more times in 6 patients for outlet stenosis or obstruction. Two patients developed a peroneal nerve paralysis associated with the rapid weight loss.
In the LAGB group, 20 patients needed revisional surgery – 12 for pouch dilation/slippage, 2 for band leakage, 2 for band erosion, and 4 for access-port problems. In the 12 patients who were reoperated for pouch dilatation/slippage, the band was repositioned in 8 cases, reduction and refixation of the pouch was performed in 3 cases, and 1 new LAGB was placed. Of these 12 pouch dilatations/slippages, 10 occurred in patients after positioning of the LAGB according to the classic peri-gastric technique and 2 after positioning with the newer pars flaccida technique.

Table 2.3 Late complications for VBG and LAGB.

Complications	Number (percentage)
VBG-group (n=50)	
Reoperations (conversion to GB)	18 (36%)
Vertical staple line disruption	15 (30%)
Narrow outlet	2 (4%)
Insufficient weight loss	1 (2%)
Incisional hernia	8 (16%)
Stenosis/obstruction (gastroscopy)	6 (12%)
Neurological problems	1 (2%)
LAGB-group (n=50)	
Reoperations	20 (40%)
Major reoperations	
Pouch dilatation/pouch slippage	12 (24%)
Band leakage	2 (4%)
Band erosion	2 (4%)
Minor reoperations	
Painful access port	2 (4%)
Infection around access port	1 (2%)

Effect on comorbidity

Following both types of gastric restrictive surgery, the number of comorbidities in both groups decreased (Table 2.4). No difference in comorbidities was observed between both groups. Associated with the weight loss, all co-morbidities tended to decrease. Joint problems, pulmonary problems and diabetes were the comorbidities which showed the greatest improvement after operation. In the patients suffering from joint problems, these resolved in 68% in the first year and in 56% after 2 years. In those with pulmonary problems, these resolved in 65% in the first year and in 76% after 2 years. Also, the effect on diabetes was impressive; at 1 year postoperatively, 83% of patients who had been treated for this comorbidity no longer required medication.

Table 2.4 Effect on comorbidity.

Comorbidity	Preoperative		1 yr. postoperative		2 yr. postoperative	
	VBG n=50(%)	LapBand n=50(%)	VBG (n=46)	LapBand (n=46)	VBG (n=48)	LapBand (n=48)
Total of patients	41[a] (82)	39 (78)	14 (30.4)[b]	18 (37.5)[b]	23 (47.9)[b]	20 (40)[b]
Joint problems	29 (58)	28 (56)	7 (15.2)[b]	10 (20.8)[b]	13 (27.1)[b]	12 (24)[b]
Pulmonary problems	8 (16)	9 (18)	3 (6.5)[c]	3 (6.3)[c]	3 (6.3)[c]	1 (2)[c]
Hypertension	10 (20	7 (14)	8 (17.4)	5 (10.4)	7 (14.6)	5 (10)
Diabetes mellitus	7 (14)	5 (10)	1 (2.2)[c]	1 (2.1)[c]	1 (2.1)[c]	1 (2)[c]
Cardiovasc. Problems	3 (6)	2 (4)	2 (4.3)	2 (4.2)	1 (2.1)	3 (6)
Hypercholesterolemia	2 (4)	2 (4)	1 (2.2)	2 (4.2)	1 (2.1)	1 (2)
Reflux-problems	2 (4)	3 (6)	0 (0)	0 (0)	0 (0)	0 (0)
Sleep-apnea	1 (2)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)
Neurological problems	0 (0)	1 (2)	0 (0)	1 (2.1)	1 (2.1)	1 (2)

[a] No. of patients; [b] $P \leq 0.001$ compared to preoperative; [c] $P \leq 0.05$ compared to preoperative.

Discussion

Morbid obesity is increasing globally. Dietary regimes and behaviour modification with or without physical activity do not sustain long-term weight loss. A treatment modality for morbid obesity is gastric restrictive surgery. Despite the increasing number of bariatric operations performed in the world, few prospective studies have been performed. In this report, the open VBG and the LAGB have been studied in a prospective randomized fashion.

It was demonstrated that both procedures can be performed with an acceptable risk, but with a relatively high reoperation rate. Three acute reoperations had to be performed in the VBG group compared to none in the LAGB group. Unfortunately, one patient (2%) died in the VBG group due to leakage of the vertical staple line directly postoperatively. This complication had not occurred in our patient population in a consecutive series of more than 200 patients in the years before the study[15]. A second patient in the VBG group died of pneumonia that he had suffered from just prior to the operation, which had not been reported to the surgeon preoperatively. Immediately postoperatively, this patient developed respiratory failure.

In contrast, in the LAGB group no patients died in the early postoperative period. Also, LAGB had a shorter mean LOS compared to VBG (3.5 vs. 6.8 days, $P<0.001$).

Morino et al.[7] compared LAGB vs. laparoscopic VBG (LVBG) in a prospective randomized trial and found similar data on days of hospitalization for the LAGB (mean of 3.7 days). Moreover, although they studied the LVBG, LOS was also similar for that technique (mean 6.6 days). The reason for shorter LOS in the

LAGB group was shorter operative time, lower morbidity and a smoother postoperative course compared to the LVBG group.
The longer LOS in the VBG group in the present study was mainly due to postoperative leakage and subsequent infections for which reoperations were necessary. These staple-line leaks are potentially the most serious immediate postoperative complications after open VBG, with a reported incidence of 1 to 3%[16]. In this report, 6% VBG leakage was found. Strangely enough, two leakages occurred in the last 3 patients of the study; however, malfunction of the stapling device could not be demonstrated.
In the LAGB group, much less immediately postoperative complications were found. The only operation-related complications were intra-operative conversions. Two LAGB procedures were converted to open (4%) due to technical problems and 1 (2%) was converted to a gastric bypass after gastric perforation. This conversion rate is comparable to other reports with a range varying between 0-6%[17,18].
The results in terms of weight loss were superior after the VBG than the LAGB, at least in the first two postoperative years. EWL of >50% after bariatric surgery is considered successful. After 1 year, an EWL of 70% was reached in the VBG group, which is comparable to that reported in the literature[19-21]. At one year postoperatively, mean weight loss was also successful in the LAGB group (53% EWL), although significantly lower than the VBG group. However, in contrast to the VBG group, in the LAGB group body weight still tended to decrease after 2 years. Morino[7] also found in two groups of patients with the same preoperative BMI, and a similar postoperative follow-up (mean 33.1 months), a higher weight loss after VBG than after LAGB.
A possible explanation for the difference in weight loss was suggested by Nilsell et al. who demonstrated that patients after a LAGB decreased weight up to 5 years postoperatively, likely due to the adjustability of the band[8]. Moreover, in contrast to our data, they found an increase in weight in the VBG group between 1 and 2 years postoperatively. In the LAGB group, optimal restriction is probably not reached within 2 years postoperatively. These data suggest that the difference in weight loss between LAGB and open VBG will diminish in time.
In our patients, all comorbidities significantly improved after surgery, in particular joint problems, diabetes and pulmonary deficiency. Despite less weight loss in the LAGB group at 1 and 2 years postoperatively, the effect on comorbidities was comparable in both groups. It is recognized that a limited amount of weight loss will lead to significant improvement in comorbidities.
Both operations showed rather disappointing long-term results with a high percentage of re-operations. In the VBG group, in 18 patients (36%) conversions to gastric bypass were necessary because of staple-line disruption (30%), too narrow outlet (4%) or insufficient weight loss (2%). Morino et al.[7] in

their randomized trial on LAGB and LVBG, found no staple-line disruptions after LVBG; however, they performed gastroplasties according to the MacLean divided technique[22]. Other studies reported high rates of staple line disruption with the Mason technique ranging from 20% to 27%[23,24], comparable to our results.

Also, 16% of our patients developed an incisional hernia. Arribas et al.[25] have demonstrated that BMI is the only patient-related factor that significantly influences the incidence of incisional hernia in morbidly obese patients. In their retrospective study of 80 morbidly obese patients operated between 1986 and 1993, an incidence of incisional hernia after gastric restrictive surgery of 24% in morbidly obese and 51% in super obese subjects was found, up to 11 years. Because our follow-up period was 2 years, these data suggest that more incisional hernias will develop in our open VBG patients, which is a strong argument for laparoscopic technique in morbidly obese patients. No incisional hernia developed in our LAGB patients.

However, our LAGB group developed many late complications. Pouch dilatation and pouch slippage were seen in 12 patients (24%). The cause of this high slippage rate is probably the positioning of the band. Dargent et al.[13] proposed the pars flaccida technique to reduce band slippage.

In this report only the last 18 bands were positioned by the pars flaccida technique (with 2 slippages – 11%) compared to 32 by the peri-gastric technique (with 10 slippages – 31%) (P=0.001). Similar slippage rates for the peri-gastric technique have been reported[26].

A further complication of LAGB was band erosion in 2 patients (4%). Others report an erosion rate of 1 to even 11%[27,28].

In summary, we report a prospective randomized clinical trial comparing open VBG and LAGB. All patients completed 2-year follow-up. Although VBG resulted in superior weight loss in the observation period, there were more severe immediate postoperative complications and the reoperationrate was significantly higher. This in combination with the advantage of the laparoscopic approach with shorter LOS and quicker return to normal daily activities, made the LAGB superior to the VBG.

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Chapter 3

Gastric myoelectrical activity in morbidly obese patients before and 3 months after gastric restrictive surgery

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Abstract

Background
Morbid obesity is often associated with gastrointestinal motor disorders. The aim of this study was to investigate gastric motility in morbid obesity, using electrogastrography (EGG) before and 3 months after gastric restrictive surgery.

Methods
Forty morbidly obese subjects (age 40.6±10.3 years, BMI 46.4±5.7 kg/m^2) were studied. Vertical banded gastroplasty (VBG) and laparoscopic gastric band (Lap-Band) operations were performed in 19 and 21 patients respectively. The following EGG-parameters were determined, both during fasting (f) and postprandially (pp): dominant frequency ($DF_{f/pp}$), dominant power ($DP_{f/pp}$), dominant frequency and power instability coefficient (DFIC and DPIC respectively) and power ratio.

Results
In the Lap-Band group, DF_{pp}, DP_{pp} and $DFIC_{pp}$ were significantly higher compared with the preprandial state, both preoperatively and 3 months postoperatively. After VBG, DF_f and $DFIC_{pp}$ were significantly lower and $DPIC_f$ significantly higher compared with the preoperative state. Furthermore, DF_{pp} and DP_{pp} were significantly higher than the preprandial values. However, in both types of operations, power ratio did not differ significantly between the preoperative and postoperative situation. Furthermore, no clear difference in EGG-parameters between both operations could be observed.

Conclusion
After gastric restrictive surgery, no major changes in gastric myoelectrical activity occurred, suggesting that if clinical motility problems occur after bariatric surgery, they are not due to gastric myoelectrical dysfunction.

Introduction

Motility of the stomach involves changes in gastric myoelectrical activity, wall movements and transit of intraluminal contents. A non-invasive technique to measure gastric myoelectrical activity is electrogastrography (EGG) using cutaneous electrodes. The gastric myoelectrical signal, derived from these cutaneous electrodes, correlates with simultaneously performed serosal measurements[1,2]. Because of new technology in data-storage and computerized EGG data analysis, this technique gained interest over the last 20 years, and the use of standardized techniques made clinical use of EGG possible[3]. Several abnormalities in EGG-recordings were described in patients with gastroparesis, functional dyspepsia and motion sickness[4-6]. Furthermore, an increasing number of studies in adults indicate an association between gastrointestinal motor disorders and EGG abnormalities[7-9].

Obesity is associated with decreased longevity and increased morbidity because of a variety of disorders and diseases such as cardiovascular diseases, type 2 diabetes, hypertension and hyperlipidemia[10]. Furthermore, morbid obesity is often associated with gastro-oesophageal motility disorders[11]. A treatment modality for morbid obesity (body mass index (BMI)≥40 kg/m^2) is gastric restrictive surgery. Several gastric restrictive surgical procedures exist which are shown to be successful in reducing body weight and obesity-related morbidity for a long period of time[12,13].

When motility disorders occur after gastric restrictive surgery, they often present as gastro-oesophageal reflux and dyspepsia[14]. However, improvement of gastro-oesophageal symptoms has also been reported after gastric restrictive surgery[15].

Because gastric restrictive surgery is performed near the gastric pacemaker area, changes in gastric myoelectric activity could be expected. This is the first prospective study to investigate the effect of two frequently used types of gastric restrictive surgery on gastric myoelectrical activity.

Patients and methods

Study design

Subjects

Forty morbidly obese patients participated in the study and were admitted to the Surgical Department of the University Hospital Maastricht to undergo gastric restrictive surgery. All patients met the inclusion criteria for operation: BMI>40 kg/m^2 or BMI>35 kg/m^2 with concomitant obesity related morbidity. All

subjects were otherwise healthy according to history, clinical examination and routine laboratory findings. Patients were randomly assigned for treatment by the laparoscopic gastric band (Lap-Band, INAMED, Santa Barbara, CA, USA (n=21)) or by vertical banded gastroplasty (VBG, n=19). The study was approved by the ethical committee of the University Hospital Maastricht. All subjects gave informed consent.

Vertical Banded Gastroplasty

In our hospital the procedure was performed as described by Mason[16] (Figure 3.1A). In short, a small pouch of the stomach of approximately 15-20 ml was created with a 4-row linear stapler (TA-90B) precisely to the angle of His, and a Dacron band of 5.0 cm in circumference, was placed through the window formed by a circular stapler (Premium Plus CEEA 31 mm, United States Surgical Corp., Norwalk, CT, USA), leaving a very small opening for food to pass from the small pouch to the remaining stomach. Because of the small capacity of the gastric pouch, the amount of ingested food causing satiety is considerably limited, leading to extensive weight loss[17].

Laparoscopic Gastric Banding

Since 1993, laparoscopic gastric banding operations have been performed (Figure 3.1B)[18,19]. The Lap-Band is made of soft silicone and equipped with an elastic balloon that can be inflated to the desired volume by means of injection postoperatively. After inflation, the outlet diameter will be reduced, leading to diminished food intake and consequently weight loss.
For definitive positioning of the band, a 15 ml calibration balloon advanced by the anaesthetist and pulled up to the gastro-oesophageal junction was placed just below the cardia. Under this balloon, the band was closed, and the ventral aspect of the greater curvature of the stomach was fixed to the pouch with three or four sutures, to ensure a stable anterior position of the band. Six weeks postoperatively, the Lap-Band was insufflated when weight loss was insufficient (<6 kg). Only one insufflation was allowed during the study period.

EGG-recording

An EGG-recording was carried out before surgery and at 3 months postoperatively. Measurements were performed after at least 8 hours fasting. After abrading the skin with alcohol and sandpaper, six cutaneous electrodes (Biotrace-HR, MSB, Singapore) were placed on specific positions on the upper abdomen[20]. A reference-electrode was placed in the supraclavicular fossa. After placement of the electrodes, one hour was allowed for stabilization of electrode-skin conduction. The next hour was defined as the fasting recording

period, after which a standardized semi-liquid test meal (Oral Impact, Novartis Nutrition, Basel, Switzerland) was given.
After the first 30 minutes postprandially, in which the physiologic postprandial frequency-dip normally has taken place[21], a postprandial period of 30 minutes was defined after which the EGG-recording was terminated. Digital amplification (EGG-Module, MMS bv, Enschede, the Netherlands) of the myoelectrical signal was applied. A high-pass frequency filter with a cut-off frequency of 15 cycles per minute (cpm) was used in order to increase the signal-to-noise ratio. A sampling frequency of 4 Hz was used. Directly after temporary storage of the data in a portable system (UPS 2020, MMS bv, Enschede, the Netherlands), the data were transferred to a desktop computer for further analysis.

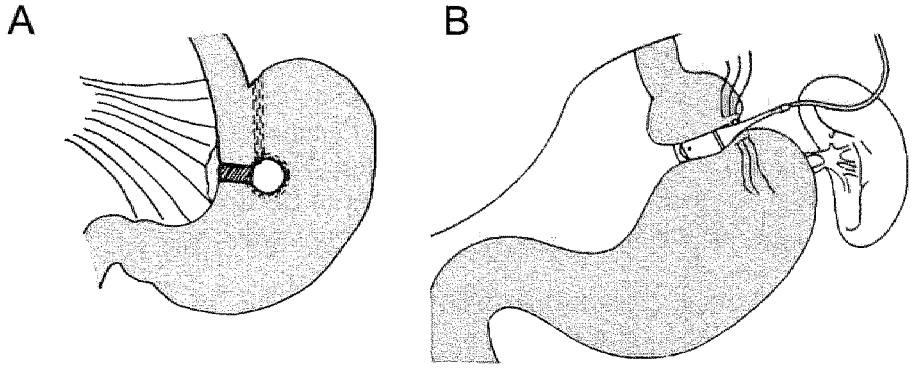

Figure 3.1 A. Vertical banded gastroplasty, B. Lap-Band operation.

EGG data analysis

From the six cutaneous electrodes at standardized positions on the upper abdomen, six unipolar and fifteen bipolar leads can be distracted. The bipolar leads were used for analysis, as these leads are less sensitive to noise artefacts. The bipolar lead with the highest amplitude was selected for further analysis[22]. The EGG-signal was visually inspected to verify that no artefacts (large positive or negative amplitude deflections) were present during the recording period. Further analysis of the EGG data was performed using dedicated software (MMS Database software, Version 7.1c), running on a personal computer.
The EGG analysis is based on the fast fourier transform (FFT) technique. A data period of 15 seconds was analyzed and termed a FFT line. For the periods of 30 minutes pre- and postprandially respectively, an average FFT line

was calculated, from which frequency and amplitude could be analyzed using running spectral analysis[23].

The following parameters were evaluated both pre- and postprandially:

1. The dominant frequency (DF) of the EGG-signal was calculated. DF is the frequency at which the power (see below) has a peak value. Normal gastric myoelectrical activity was defined as a DF between 2.6 and 3.7 cpm[24-26]. Frequencies <2.5 and >3.7 cpm were denoted as bradygastria and tachygastria, respectively.
2. The power (or amplitude) of DF was determined. The amplitude of the cutaneous signal is assumed to be a function of the velocity of propagation of the myogenic signal. Changes in EGG amplitude run parallel with changes in the intensity of electrical response activity and are suggested to reflect antral contraction amplitude[20].
3. The stability of DF, expressed as the instability coefficient of the dominant frequency (DFIC) was calculated. The DFIC is calculated by dividing the standard deviation of DF during a specific time-period (pre- or postprandially) by the mean DF during that time-interval.
4. The stability of the power of DF, expressed as the instability coefficient of the dominant power (DPIC), was calculated. The DPIC is calculated by dividing the standard deviation of the power of DF during a specific time-period (pre- or postprandially) by the mean dominant power (DP) during that time-interval.
5. Power ratio was determined. The absolute value of EGG power is influenced by several factors (e.g., skin conductance, distance between electrodes and the wall of the stomach, variable shape of the stomach, etc.). Hence, EGG power changes should only be evaluated as relative changes. The power ratio is the ratio of the postprandial to fasting EGG power values.

Statistical analysis

Data are given as mean ± standard deviation. Statistical analysis was performed non-parametrically and two-sided. Data from different recording periods in the same subject were analyzed using the Wilcoxon signed ranks test. The Mann-Whitney U test was used to analyze differences between groups. A P-value <0.05 was denoted significant.

Results

Study group

Table 3.1 summarizes the characteristics of the 40 subjects studied. Of the patients, 19 underwent the VBG operation and 21 the Lap-Band operation. No significant difference between the two patient groups was observed regarding gender, age and BMI preoperatively. Three months postoperatively, BMI was significantly more decreased in the VBG operated group compared with the Lap-Band group.

Table 3.1 Clinical characteristics of the study group.

Variable	Total study group n=40	Lap-Band-group n=21	VBG-group n=19
Age	40.6 ± 10.3	41.1 ± 10.7	40.2 ± 10.2
Sex (male/female)	8/32	5/16	3/16
BMI preoperative	46.4 ± 5.7	46.5 ± 5.3	46.3 ± 6.2
BMI 3 months postoperative	40.0 ± 5.6	41.9 ± 4.9	37.8 ± 5.5

Age and BMI are presented as mean and standard deviation.

Gastric myoelectrical activity in morbidly

Obese subjects

The preoperative EGG parameters obtained from the entire group of morbidly obese subjects during the pre- and postprandial periods are reported in Table 3.2. The DF after the postprandial semi-solid test meal was significantly higher compared with preprandial DF ($P<0.01$). Furthermore, ingestion of the semi-liquid test meal was associated with a significant increase in DP ($P<0.001$). Accordingly, the power ratio was >1. DFIC significantly decreased post-prandially, whereas DPIC did not differ.

Table 3.2 EGG parameters in all morbidly obese subjects preoperatively.

Variable	Preprandial	Postprandial	*P*-value
DF (cpm)	2.96 ± 0.22	3.03 ± 0.24	$P<0.01$
DP	1532 ± 845	2530 ± 1556	$P<0.001$
DFIC	0.086 ± 0.05	0.076 ± 0.05	$P<0.05$
DPIC	0.26 ± 0.09	0.28 ± 0.1	$P=0.390$

DF=dominant frequency; DP=dominant power; DFIC=Instability coefficient of dominant frequency; DPIC=Instability coefficient of dominant power.

Effect of Lap-Band and VBG on gastric myoelectrical activity

As shown in Table 3.3, no changes in preprandial EGG parameters were observed 3 months after the Lap-Band operation. However, postprandial DF, DP and DFIC were significantly higher compared with the preprandial state, both preoperatively ($P<0.05$) and 3 months postoperatively ($P<0.05$).
After VBG (Table 3.4), the preprandial DF was significantly lower ($P<0.01$) 3 months postoperatively compared with the preoperative state. Next to this, the preprandial DPIC at 3 months postoperatively was significantly higher ($P<0.05$) and the postprandial DFIC significantly lower ($P<0.05$) compared with the preoperative state. Furthermore, the postprandial DF and DP were significantly higher ($P<0.01$) than the preprandial values. However, the power ratio did not differ significantly between the preoperative and postoperative situation in both types of operations.
When the effects of both types of operation on the gastromyoelectrical activity were compared, no significant difference could be observed for the different EGG parameters, except for the 3 months postoperative preprandial DF and DFIC in the VBG group which were significantly changed compared with preoperative values ($P<0.01$ and $P<0.05$ respectively). However, the change between preprandial and postprandial DF, DP, DFIC and DPIC at both time-points between both types of operation, did not differ significantly.

Table 3.3 Effect of Lap-Band operation on EGG parameters.

Lap-Band-group (n=21)	Preprandial	Postprandial	*P*-value
Preoperative			
DF (cpm)	2.96 ± 0.26	3.04 ± 0.24	$P<0.01$
DP	1744 ± 870	2745 ± 1455	$P<0.001$
DFIC	0.09 ± 0.05	0.07 ± 0.04	$P<0.05$
DPIC	0.27 ± 0.10	0.27 ± 0.09	NS
Power Ratio	-	1.88 ± 0.84	
3 months postoperative			
DF (cpm)	3.00 ± 0.17	3.13 ± 0.30	$P<0.01$
DP	1831 ± 1366	2449 ± 1482	$P<0.05$
DFIC	0.07 ± 0.04	0.09 ± 0.11	NS
DPIC	0.27 ± 0.09	0.31 ± 0.23	NS
Power Ratio	-	1.84 ± 1.12	

DF=dominant frequency; DP=dominant power; DFIC=Instability coefficient of dominant frequency; DPIC=Instability coefficient of dominant power; NS=not significant.

Table 3.4 Effect of VBG on EGG parameters.

VBG-group (n=19)	Preprandial	Postprandial	*P*-value
Preoperative			
DF (cpm)	2.95 ± 0.18	3.01 ± 0.24	NS
DP	1300 ± 771	2292 ± 1668	*P*<0.001
DFIC	0.083 ± 0.05	0.087 ± 0.06	NS
DPIC	0.25 ± 0.09	0.28 ± 0.12	NS
Power Ratio		1.88 ± 0.66	
3 months postoperative			
DF (cpm)	2.73 ± 0.30[a]	3.13 ± 0.75	*P*<0.01
DP	1534 ± 887	2537 ± 1907	*P*<0.01
DFIC	0.105 ± 0.09	0.054 ± 0.03[b]	NS
DPIC	0.29 ± 0.06[b]	0.29 ± 0.09	NS
Power Ratio	-	1.98 ± 1.10	

DF=dominant frequency; DP=dominant power; DFIC=Instability coefficient of dominant frequency; DPIC=Instability coefficient of dominant power; NS=not significant; [a] *P*<0.01 compared to preoperative; [b] *P*<0.05 compared to preoperative.

Discussion

In this report, we demonstrate that in morbidly obese subjects, DF and DP significantly increase after a test meal. These observations are in line with data of others, who reported an increase in DF and DP after a test meal in normal weight adults[27-29]. DPIC in the morbidly obese patients significantly decreased postprandially, indicating a decrease in the variation of DF within the normal range (2.4-3.7 cpm) after a semi-liquid test meal. Although equivocal data exist in healthy volunteers[8,30,31], our data were in line with results of Pfaffenbach et al.[29] who demonstrated in healthy individuals a postprandial decrease in DFIC. Next to DFIC, DPIC was also measured. Similar to results in healthy subjects, morbidly obese subjects did not show a significant difference between preprandial and postprandial DPIC[8,30,31]. These data suggest that contractility does not change after a semi-liquid test meal in morbidly obese and normal weight subjects.

The effect of two types of bariatric surgical techniques on gastric myoelectrical activity, as measured by electro-gastrography, was evaluated. Only after VBG, DFIC was significantly decreased postprandially compared with preprandial data. Furthermore, after VBG a significant decrease of the preprandial DF and a higher DPIC compared with preoperative values could be observed. The latter suggests an increase in gastric contractility due to VBG, because DPIC is generally assumed to mirror myogastric contractility[8]. Nevertheless, the power ratio did not differ between the preoperative and postoperative situation, implicating that the relative increase in power because of a semi-solid test meal is not influenced by VBG. In addition, a significant difference between both

types of operation postoperatively concerning changes in gastric myoelectrical activity was not observed. These data indicate that these two types of gastric restrictive surgery do not induce major changes in gastric myoelectrical activity. As far as we know, only Halpern et al.[32] described the effect of bariatric surgery on gastric electrical activity. In their study, 18 patients were studied, of which six underwent Roux-en-Y gastric bypass, six patients gastric baffle and six patients silastic ring vertical gastroplasty, respectively. In these patients, three pairs of serosal bipolar electrodes were implanted on the stomach at the time of operation. Gastric electrical activity was measured perioperatively as well as 8 days postoperatively. Gastric myoelectrical activity changed only in the gastric fundus after operation compared with preoperative values. The question arose what could be the cause of this change in fundal gastric myoelectrical activity postoperatively. As a possible explanation, the authors speculated that the crushing produced by placing the stapling device across the entire gastric fundus interrupts an already weakly propagated electrical activity in the gastric fundus. On the other hand, no significant difference in antral electrical activity could be observed in this study. Taking into consideration that other authors have demonstrated that the site of origin of the electrical activity in the human stomach is mainly located in the gastric body[33-35], these data suggest that the staples used for gastric restrictive surgery probably do not interfere with antral motility of the stomach.
It should be mentioned, however, that the follow-up period in the latter study was limited[32] and probably heavily influenced by the surgical trauma. In order to exclude a direct effect of the surgical trauma on the EGG-measurements and to cover possible adaptation mechanisms of the stomach, in the present study data were obtained at 3 months postoperatively.
We also studied the effect of the Lap-Band-operation on gastric electrical activity and found that the electrical activity did not significantly change 3 months after operation. These data suggest that placement of a gastric band around the uppermost part of the stomach probably does not interfere with antral motility, because no dysrhythmias were observed.
However, EGG only measures propagation of antral electrical activity. Using EGG, gastric myoelectrical activity of the fundus cannot be measured. Theoretically, it is still possible that fundal electrical activity is disturbed. Furthermore, changes in fundic relaxation also cannot be excluded after bariatric surgery.

In summary, we found that 3 months after gastric restrictive surgery no major changes in gastric myoelectrical activity occurred. These data suggest that myoelectrical dysfunction of the antrum is not involved in motility disorders in morbidly obese patients after bariatric surgery.

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Chapter 4

Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals

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Abstract

Objective
To study whether an increase of plasma leptin concentrations, as observed in the case of increased body weight, is associated with an inflammatory state.

Subjects
Sixty-three healthy subjects with body mass index (BMI) ranging from 20 to 61 kg/m^2.

Measurements
Plasma concentrations of leptin, the inflammatory parameter soluble TNFα receptors (TNFR55 and TNFR75), the acute phase proteins lipopolysaccharide binding protein (LBP), serum amyloid A (SAA), α1-acid glycoprotein (AGP), C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and the anti-inflammatory soluble Interleukin-1 decoy receptor (sIL-1RII) were measured.

Results
As expected, BMI correlated significantly with leptin ($r=0.823$, $P<0.001$), but also with all acute phase proteins, both soluble TNF receptors and PAI concentrations. After correction for BMI and sex, no significant correlation between leptin and the acute phase proteins was seen. Interestingly, however, leptin strongly correlated with both TNF-receptors ($r=0.523$, $P<0.001$ for TNFR55 and $r=0.438$, $P<0.001$ for TNFR75).

Conclusion
This study shows the development of a pro-inflammatory state with increasing body weight. The BMI independent relationship between leptin and both soluble TNF-receptors is consistent with a regulatory role for leptin in the inflammatory state in morbidly obese subjects.

Introduction

Leptin, the product of the Ob gene[1,2], is considered to be involved in satiety regulation and obesity. Leptin is primarily expressed in adipose tissue[3] and studies in mice show a central role for leptin in food intake and regulation of energy balance[4–7]. Leptin-deficient mice (ob/ob mice) or leptin-receptor deficient mice (db/db mice) develop obesity[8,9]. Administration of leptin to ob/ob mice increases energy expenditure, decreases body weight and normalises hyperglycaemia, insulin resistance and hyperinsulinaemia[10–12]. In addition, it has been demonstrated that the administration of exogenous leptin to ob/ob mice prevents LPS and TNFα-induced lethality[13,14]. Next to this, other reports also indicate that leptin has immunoregulatory and immunoprotective effects[14-16].

Human obesity is characterised by increased plasma leptin concentrations. Furthermore, obesity is associated with decreased longevity and increased morbidity due to a variety of disorders and diseases such as cardiovascular diseases, type 2 diabetes mellitus, hypertension and hyperlipidaemia[17].

Several studies suggest a pathophysiological role of inflammatory markers in the development of insulin resistance and cardiovascular diseases[18]. It has been demonstrated *in vivo* and *in vitro* that TNFα plays a role in mediating insulin resistance[19–23]. Overall, it appears that upregulation of inflammatory markers is involved in the development of obesity-related diseases.

Several studies have suggested an enhanced inflammatory state in morbidly obese patients as evidenced by increased plasma concentrations of cytokines and acute phase proteins[24–28] without direct clinical evidence of acute or chronic inflammation in these patients. The underlying mechanism of these elevated plasma concentrations of inflammatory markers and their role in the pathophysiology of morbid obesity is still unknown. We hypothesized that leptin is involved in the induction of this enhanced inflammatory state in obese subjects. In order to study this, we investigated the relation between body mass index (BMI), leptin and inflammatory markers in a group of subjects ranging in BMI from 20 to 61 kg/m^2.

Subjects and methods

In total 63 subjects were included in the study. These 63 subjects were selected to create a study population with a wide range of BMIs. Thirty-eight subjects were admitted to the Surgical Department of the University Hospital Maastricht to undergo surgical treatment for morbid obesity. The majority of these patients (n=32) underwent a primary operation for morbid obesity

(vertical banded gastroplasty (VBG) or laparoscopic adjustable gastric banding (Lap-Band), BMI range from 37 to 61 kg/m^2). The remaining six patients underwent a gastric bypass as revision surgery. Revision was necessary because of excessive weight loss (n=1) or weight regain as a result of a staple line disruption (n=5; BMI range 23-46 kg/m^2). Twenty-five healthy subjects were included, matched for gender and age. All subjects were otherwise healthy according to history, clinical examination and routine laboratory findings. In particular, none of the studied subjects had any evidence of acute or chronic inflammatory disease. Of the morbidly obese patients two had type 2 diabetes mellitus and one had hypertension. No patient had signs of cardiovascular disease. Characteristics of the subjects are presented in Tables 4.1 and 4.2.

Blood samples were collected after at least 8 h fasting using evacuated blood collection tubes containing EDTA. In the patient group blood samples were taken at the day of admission to the hospital, one day before surgery. The blood samples were immediately put on melting ice, and plasma was prepared by centrifugation at 1,400*g* for 10 min at 4°C. The supernatant was centrifuged at 2,700*g* for 10 min at 4°C and stored in aliquots at -80°C.

All participants gave written informed consent and the study was approved by the local ethical committee of the Academic Hospital Maastricht.

Table 4.1 Characteristics of the study group.

Variable	Study group (n=63)	Male (n=11)	Female (n=52)
Age[a]	35 ± 6.89	33 ± 7.32	36 ± 6.79
BMI[b] (kg/m^2)	37 (20–61)	34 (24-48)	39 (20-61)

[a] Age is presented as mean and standard deviation; [b] BMI is presented as median and range.

Table 4.2 Characteristics of the study group.

Range BMI	Number of subjects	Male	Female
BMI<40 kg/m^2	35	8	26
BMI≥40 kg/m^2	28	3	26

Reagents and materials

Monoclonal antibodies (mAbs) specifically directed against soluble TNFα receptor 55 (TNFR55) and soluble TNFα receptor 75 (TNFR75) were obtained as described elsewhere[29]. Polyclonal rabbit antisera anti-TNFR55 and anti-TNFR75 were obtained by immunising rabbits with TNFR55 and TNFR75, respectively.

Human recombinant lipopolysaccharide binding protein (LBP), used as standard, was produced by transfected Chinese Hamster Ovary (CHO) cells, kindly provided by Dr. P. Tobias (Research Institute of Scripps Clinic, La Jolla, CA). Polyclonal antibodies to human LBP were obtained by immunising rabbits with human LBP. A serum amyloid A (SAA) immunoassay was kindly provided by Dr. P.C. Limburg (Department of Rheumatology, University Groningen, The Netherlands). Human C-reactive protein (CRP) was obtained from Dade Behring (Deerfield, Illinois); rabbit anti-human CRP and rabbit anti-human CRP-HRP were purchased from DAKO (Glostrup, Denmark).
Human α1 acid glycoprotein (AGP) was obtained from Sigma (St Louis, MO) and rabbit anti-human AGP from DAKO (Glostrup, Denmark). Peroxidase-conjugated streptavidin was purchased from Dakopatts (Glostrup, Denmark) and TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate from Kirkegaard & Perry Lab (Gaithersburg, MD). Immuno maxisorp plates (Nunc, Roskilde, Denmark) were used for ELISAs.

Immunoassays

Plasma concentrations of both soluble TNFα receptors, LBP, SAA, CRP and AGP concentrations were measured using sandwich ELISAs. TNFR55, TNFR75 and LBP were detected as described elsewhere[29,30]. These ELISAs had a lower detection limit of approximately 100 pg/ml. Plasma CRP and AGP concentrations were measured using ELISAs developed in our institute. ELISA plates were coated overnight with polyclonal anti-human CRP and AGP respectively. Diluted plasma samples (1:500 for CRP and 1:200,000 for AGP) and a standard dilution series with human rCRP and rAGP, respectively, were added to the plate. Detection for CRP occurred with a HRP-labelled polyclonal rabbit anti-human CRP followed by substrate. Detection for AGP was carried out with a biotinylated polyclonal rabbit anti-human AGP IgG, followed by peroxidase-conjugated streptavidin and substrate. SAA was quantified as described previously[19]. The detection limit for the SAA assay was 100 pg/ml.
Plasma leptin concentrations were measured using a commercially available leptin ELISA (BioVendor, Brno, Czech Republic). The detection limit of this assay is 0.2 ng/ml.
PAI-1 concentrations were measured using an Elisa kit, kindly provided by Dr. T. Kooistra, Leiden, The Netherlands. In short, microtitre strip plates were coated with a highaffinity mAb against PAI-1. Detection for PAI-1 was carried out using a HRP-labelled antibody conjugate followed by TMB.
For sIL-1RII detection, plates were coated with mAbs against sIL-1RII. The reagents were kindly provided by Hbt (Uden, The Netherlands) in the context of a European Commission grant (grant BIO4-CT97-2107). After adding the

samples, detection was carried out with a biotinylated polyclonal rabbit anti-human sIL-1RII, followed by peroxidase-conjugated streptavidin and substrate. All plasma samples were analysed in the same run. When plasma concentrations exceeded the upper detection limit of the assay, samples were additionally diluted and analysed in a separate run with an overlap to correct for inter-assay variation. The intra- and inter-assay coefficients of variance of the various assays were all <10%.

Statistical analysis

Pearson correlation coefficients were computed between the parameters under investigation. In addition, partial correlation coefficients were calculated for leptin and inflammatory markers, adjusted for BMI and gender.
Group comparisons were performed by unpaired Student's t-test. Statistical analyses were done using the SPSS 10.0.5 statistical package. All *P*-values are two-tailed and a value of $P<0.05$ was considered statistically significant.

Results

Study group

Tables 4.1 and 4.2 summarise the characteristics of the 63 subjects studied. The population showed a distribution of BMI between 20 and 61 kg/m^2 from lean to morbid obese subjects. The study group was divided into two groups to separate morbidly obese subjects (BMI≥40 kg/m^2) from non-morbidly obese subjects (BMI<40 kg/m^2).

BMI and leptin

Plasma leptin concentrations in the overall study population ranged from 0.8 to 82 ng/ml. These concentrations correlated strongly with BMI ($r=0.82$, $P<0.001$, Figure 4.1). Morbidly obese subjects had a mean plasma leptin concentration of 52.7±19.8 ng/ml. In these morbidly obese patients, leptin also significantly correlated with BMI ($r=0.61$, $P<0.001$). Lean subjects (BMI<25) showed a mean plasma leptin concentration of 7.6±4.8 ng/ml.

BMI and acute phase proteins

Previous studies suggested an increase of markers of the acute phase reaction in morbid obesity[27,31]. To further evaluate the presence of proteins characteristic for the acute phase reaction, plasma concentrations of the pentraxin CRP, AGP, SAA and LBP were measured. As depicted in Table 4.3,

CRP and SAA showed the most marked increase in plasma concentrations in relation to BMI. CRP concentrations in morbidly obese subjects were approximately 20 times the normal values and ranged from 0.13 to 56.6 μg/ml (median 8.2 μg/ml). These values correlated significantly with BMI concentrations (r=0.547, $P<0.001$).

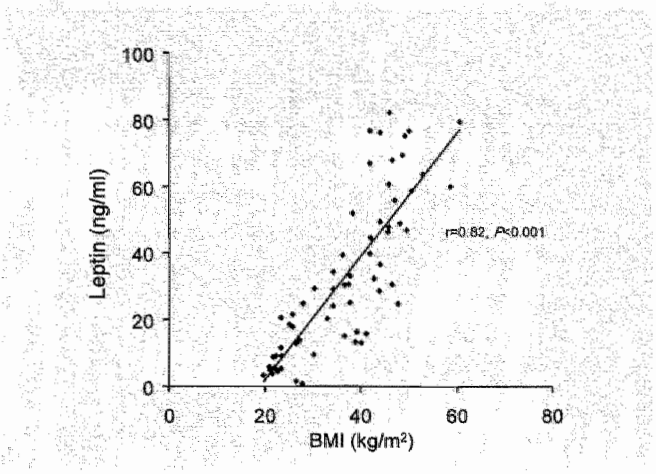

Figure 4.1 Leptin correlated significantly with BMI. The Pearson correlation coefficient is given.

Plasma concentration of SAA increased approximately 10 times the normal values and ranged from 0.03 to 6.01 ng/ml (median 1.2 μg/ml) and also correlated significantly with BMI (r=0.371, $P<0.01$). Furthermore both CRP and SAA correlated with leptin (r=0.530, $P<0.001$ and r=0.356, $P<0.01$, respectively).

Plasma concentrations of AGP and LBP revealed a weaker, but still significant correlation with BMI. In the studied group AGP ranged from 0.34 to 1.96 mg/ml and showed a significant correlation with BMI (r=0.284, $P<0.05$). Lean subjects showed a mean plasma AGP concentration of 0.88±0.2 mg/ml; in morbidly obese subjects mean plasma AGP concentrations were approximately 1.3 times higher (1.12 ±0.4 mg/ml).

LBP ranged from 3.68 to 45.82 μg/ml (in lean subjects mean 10.7±3.3 μg/ml, in obese subjects 14.9±6.6 μg/ml, 1.5 times higher compared to lean subjects; correlation with BMI: r=0.275, $P<0.05$). AGP and LBP correlated also with leptin (r=0.354, $P<0.01$ for AGP and r=0.336, $P<0.01$ for LBP, respectively).

BMI and plasminogen activator inhibitor-1

The above-mentioned increase in CRP, SAA, AGP and LBP is supposed to be caused by enhanced production of these proteins by hepatocytes. Another

protein which is associated with an acute phase reaction is plasminogen activator inhibitor-1 (PAI-1). This protein is mainly produced by endothelium. Measurement of PAI-1 may give additional information concerning the involvement of extra hepatic processes in the enhanced inflammatory process in morbidly obese patients. Plasma concentrations of PAI-1 were measured in the study group and correlated with BMI. PAI-1 concentrations ranged from 0.12 to 113.44 ng/ml (median 14.92 ng/ml) and significantly correlated with BMI ($r=0.419$, $P<0.001$), suggesting an enhancement of extrahepatic inflammatory markers with increasing body weight.

Table 4.3 Cross-table of the correlations of all measured parameters[a].

	BMI	LEPTIN	CRP	AGP	SAA	LBP	PAI	SIL1RII	TNFR55
LEPTIN	0.823[b]								
CRP	0.547[b]	0.530[b]							
AGP	0.284[d]	0.354[c]	0.361[c]						
SAA	0.371[c]	0.356[c]	0.658[b]	0.420[b]					
LBP	0.275[d]	0.336[c]	0.466[b]	0.396[b]	0.365[c]				
PAI	0.419[b]	0.382[c]	0.159	0.236	0.109	-0.081			
SIL1RII	0.323[d]	0.227	0.305[d]	0.292[d]	0.102	0.257[d]	0.367[c]		
TNFR55	0.421[b]	0.598[b]	0.321[d]	0.541[b]	0.248[d]	0.205	0.406[b]	0.227	
TNFR75	0.274[d]	0.447[b]	0.297[d]	0.524[b]	0.252[d]	0.344[c]	0.247	0.334[c]	0.843[b]

[a] Data are expressed as correlation coefficient, measured by Pearson correlation analysis; [b] $P\leq0.001$; [c] $P<0.01$; [d] $P<0.05$.

BMI and soluble TNFα receptors

In order to further evaluate the relation of a possible chronic systemic inflammatory response with increasing leptin concentrations, soluble TNF receptors (TNFR55 and TNFR75) were measured. Plasma concentrations of TNFR55 and TNFR75 ranged from 0.30 to 0.99 ng/ml and 0.45 to 1.94 ng/ml, respectively. A significant positive correlation was found between BMI and TNFR55 ($r=0.421$, $P<0.001$). Lean subjects showed a mean plasma concentration of 0.52±0.1 ng/ml, obese subjects 0.69±0.17 ng/ml. TNFR75 was, to a lesser extend, also significantly related to BMI ($r=0.274$, $P<0.05$). Lean subjects had a mean plasma concentration of 1.01±0.21 ng/ml; obese subjects 1.22±0.37 ng/ml.

BMI and sIL-1RII

Inflammation is associated with an increase of pro- and anti-inflammatory activity. In order to study the effect of increasing BMI and activation of an inflammatory response, the soluble IL-1 decoy receptor (sIL-1RII) was

measured because it is demonstrated to have anti-inflammatory properties. Plasma concentrations of sIL-1RII ranged from 0.93 to 5.6 ng/ml (median 0.93 ng/ml) and significantly correlated with BMI (r=0.323, $P<0.01$).

Leptin, TNF-Rs and acute phase proteins

In order to study the direct effect of leptin on inflammatory markers, partial Pearson correlations, corrected for BMI (an indirect measure of fat mass) and gender, were performed. The correction for gender was performed because gender and leptin correlated, independently of BMI, significantly with each other ($P<0.001$). As depicted in Table 4.4, after correction, no correlation between leptin and the acute phase proteins was demonstrated, suggesting that the relationship between leptin and the acute phase response is actually a reflection of fat mass. In a multiple regression analysis including BMI, leptin and gender as independent variables, only BMI was a significant independent determinant of CRP ($P=0.045$). However, leptin was found to be correlated with both TNF-Rs (r=0.529, $P<0.001$ for TNFR55 and r=0.438, $P<0.001$ for TNFR75; Figure 4.2A) despite correction for BMI and sex. Moreover, when the studied group was divided into two subgroups and corrected for BMI and sex (BMI≥40 and BMI<40) leptin strongly correlated in the group BMI≥40 with both TNF-Rs (r=0.717, $P<0.001$ for TNFR55 and r=0.589, $P=0.002$ for TNFR75). However, in the group BMI<40, leptin did not correlate with TNFR55 and TNFR75 (r=0.294, $P=0.096$ and r=0.151, $P=0.402$, respectively).
These correlations between leptin and the plasma concentrations of both TNF-Rs are consistent with an enhancement of these inflammatory markers by leptin, particularly in morbidly obese subjects. In line with an enhanced inflammatory response, the concentrations of TNFR55 and TNFR75 also correlated strongly with each other (r=0.83, $P<0.001$, Figure 4.2B).

Table 4.4 Correlation between leptin and the inflammatory mediators, after correction for BMI and sex[a].

	LEPTIN	TNFR55	TNFR75	CRP	AGP	LBP	SAA	PAI-1
TNFR55	0.527[b]							
TNFR75	0.438[b]	0.834[b]						
CRP	0.081	0.113	0.180					
AGP	0.20	0.484[b]	0.483[b]	0.241				
LBP	0.153	0.098	0.288[d]	0.374[c]	0.335[c]			
SAA	-0.01	0.103	0.164	0.565[b]	0.341[c]	0.272[d]		
PAI-1	0.209	0.297[d]	0.165	-0.045	0.164	-0.198	-0.003	
sIL-1RII	0.086	0.124	0.296	0.247	0.269[d]	0.245	0.053	0.213

[a] Data are expressed as partial Pearson correlation coefficient, controlled for BMI and sex;
[b] $P\leq0.001$; [c] $P<0.01$; [d] $P<0.05$.

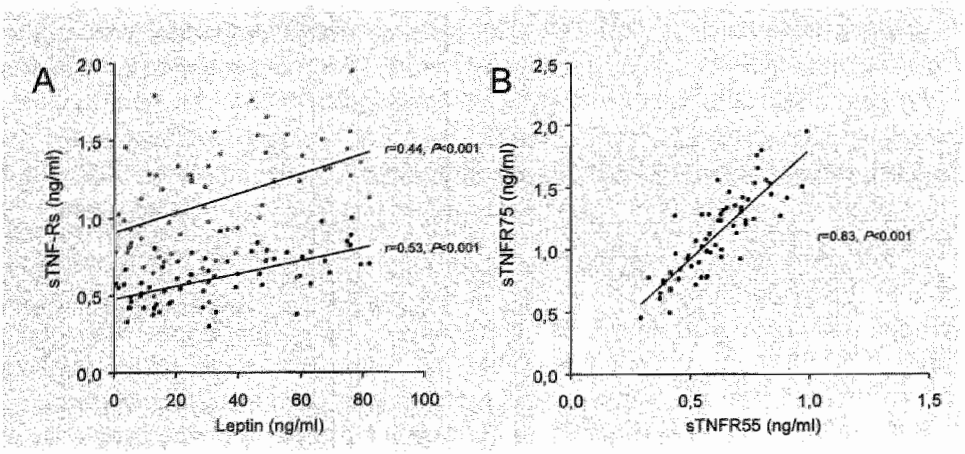

Figure 4.2 Significant correlation between leptin and both TNF-Rs. The effect of BMI and sex on these parameters was excluded by performing partial correlation analysis. A significant correlation was found between leptin and both TNF-Rs after correction for BMI and sex (A). Both TNF-Rs also strongly correlated with each other after correction for BMI and sex (B). ∘ = sTNFr75, • = sTNFr55.

Discussion

This study examines the association of BMI, leptin and the development of an inflammatory state in obese subjects. A number of inflammatory parameters were measured to characterise the acute phase type of response in the study population. Significant correlations between BMI and leptin, CRP, SAA, LBP and AGP were found. The highest correlations were found between BMI and leptin, followed by BMI and CRP, BMI with SAA, BMI with LBP and BMI with AGP respectively. These data are supported by the observations of Yudkin et al.[28].

Next to the acute phase proteins, TNFR55 and TNFR75 were measured. It is known that during experimental endotoxemia, TNF-Rs levels are significantly increased and remain, in contrast to systemic TNFα, elevated for a longer period of time[32–34]. Therefore, soluble TNFα receptor levels appear to be of value in characterising an inflammatory response[35,36]. Moreover, in obese subjects increased plasma concentrations of both TNF-Rs were reported[37], another argument to measure TNF-Rs. Corica et al. described a positive correlation between BMI and TNFR55[38], whereas others reported an increase of TNFR75 with increasing body weight[39]. In the present study we observed an increase in plasma concentrations of both TNF-Rs with increasing BMI. Moreover, the association between leptin and both TNF-Rs was highly significant. Next to this, the acute phase protein concentrations also correlated

strongly with leptin. These observations suggest a possible role for leptin in the acute phase response observed with increasing body weight and could be an indication for a more central role of leptin in the inflammatory process in these patients.

A more central role for leptin in inflammatory processes is also supported by recent studies indicating a regulatory effect of leptin on the immune response. Firstly, leptin is able to induce upregulation of pro-inflammatory cytokines, both *in vivo* and *in vitro*[40]. Secondly, leptin-deficient mice show increased lethality after LPS challenge which could be prevented by leptin suppletion[13,14] and thirdly, leptin modulates the T-cell immune response by increasing Th1- and suppressing Th2-cytokine production[15]. These findings indicate a role for leptin in the immune response. In this context, the elevated plasma leptin concentrations in morbidly obese patients may enhance constitutive immunological stimuli, leading to increased concentrations of acute phase proteins and other inflammatory markers, characteristic for a chronic inflammatory state.

However, because BMI also strongly correlates with the different inflammatory parameters measured, these data were corrected for BMI and sex. After correction for BMI and sex, no correlation between leptin and the acute phase proteins was found, indicating that, besides leptin, fat mass itself is also involved in the acute phase response. Nevertheless, leptin correlated independently of BMI with both TNF-Rs, which is consistent with a role of leptin in the enhancement of these inflammatory markers.

Most interestingly, when the total group studied was divided into two groups (non-morbidly obese subjects (BMI<40 kg/m^2) and morbidly obese subjects (BMI≥40 kg/m^2)) leptin and both TNF-Rs correlated strongly with leptin in the BMI≥40 group, whereas such a correlation was not found in the BMI<40 group. The reason for this apparent discrepancy of the correlation between leptin and both TNF-Rs in these groups remains to be resolved. Interestingly, we have also found significant independent correlations between leptin and TNF-Rs in cachectic patients. Cachexia is defined by loss of lean tissue mass. However, in the studied patient-group a loss of lean tissue mass was present despite preservation of fat mass[41].

During an inflammatory process acute phase protein production is assumed to be enhanced in hepatocytes. However, whether hepatocytes are responsible for the measured increase of the acute phase proteins with increasing BMI remains to be elucidated. Recent reports indicate that other cells besides hepatocytes can also produce acute phase proteins during an inflammatory process. For example, human intestinal epithelial cells were demonstrated to produce LBP and SAA after stimulation with TNFα, IL-6 or Il-1β[42]. To evaluate whether the increased inflammatory markers, responsible for the enhanced acute phase proteins, will stimulate not only hepatocytes but other cells as well,

PAI-1, a product of endothelial cells, was measured. It is known that plasma concentrations of PAI-1 increase during an inflammatory response[43,44]. In this report PAI-1 concentrations were found to be related to BMI and TNFR55, suggesting that the enhanced inflammatory response in obese subjects is not restricted to the liver. Furthermore, a positive correlation between leptin and PAI-1 concentrations was seen.

In order to further evaluate the observed systemic inflammatory process in obese subjects, plasma concentrations of the anti-inflammatory mediator sIL-1RII were also measured. During an inflammatory process, the production and release of this anti-inflammatory soluble receptor is shown to be enhanced[45]. In the present study a weak, but significant, correlation between plasma concentrations of sIL-1RII and BMI was found, suggesting that not only a pro-inflammatory, but also an anti-inflammatory process is enhanced with an increase in body weight.

Several recent studies suggest a relation between inflammatory markers and the development of obesity-related disorders, like cardiovascular diseases and type 2 diabetes mellitus[18,20,46–48]. Elevated plasma concentrations of PAI-1, but also CRP and SAA are related with an increased risk for cardiovascular diseases[49–52]. Furthermore, it is reported that TNFα plays a major role in the development of insulin resistance[20,47,48]. The present study demonstrates that with increasing body weight a systemic inflammatory state develops and that leptin specifically correlated with both TNF-Rs in morbidly obese subjects, suggesting that leptin plays a role in the enhancement of the inflammatory activity in these morbidly obese subjects, eventually leading to obesity related disorders.

The origin of the pro-inflammatory cytokines, responsible for the induction of the inflammatory state in obese subjects, could be adipose tissue. It was previously shown that leptin as well as TNFα and IL-6 are produced in adipose tissue and increase with increasing body weight[19,20,31,38,40,53–58]. Next it was recently demonstrated in animal studies that adipocytes express the receptors for microbial toxins such as LPS (the Toll-like receptor-2 and 4), suggesting an important role for adipose tissue in the immune response[59].

In summary, the present study shows the enhancement of an inflammatory state with increasing plasma leptin concentrations due to increased body weight. The strong correlation of the inflammatory markers with leptin, particularly in morbidly obese subjects, is consistent with a role for leptin in the regulation of the inflammatory state in these subjects.

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Chapter 5

Macrophage inhibitory factor, plasminogen activator inhibitor-1, other acute phase proteins, and inflammatory mediators normalize as a result of weight loss in morbidly obese subjects treated with gastric restrictive surgery

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Abstract

Background
Obesity is demonstrated to be associated with an enhanced inflammatory state, which is suggested to be a cause for the development of obesity-related morbidity. It was hypothesized that a decrease in body weight in morbid obese subjects would lead to a reduction of the inflammatory state in these subjects.

Methods
Weight loss was achieved by gastric restrictive surgery in 27 morbidly obese patients. Preoperative as well as 3-, 6-, 12-, and 24-month postoperative plasma concentrations of inflammatory mediators macrophage inhibitory factor, plasminogen activator inhibitor-1, lipopolysaccharide binding protein, α1 acid glycoprotein, C-reactive protein, soluble TNFα receptors 55 and 75, and leptin were measured.

Results
Macrophage inhibitory factor levels remained low normal for 6 months, during weight loss, after which they significantly increased to normal levels at 24 months postoperatively. The other inflammatory mediators remained elevated up to minimally 3 months postoperatively; thereafter they decreased significantly. Both TNFα receptors remained elevated up to at least 12 months postoperatively to decrease significantly at 2 year postoperatively.

Conclusions
This study demonstrates that during weight loss, after gastric restrictive surgery, inflammatory mediators remain elevated for at least 3 months postoperatively, suggesting initially an ongoing inflammatory state. However, 2 year after surgery, the inflammatory mediators reach near normal values. These findings may be an explanation for the reduced comorbidity seen in morbidly obese patients after gastric restrictive surgery.

Introduction

Obesity is associated with decreased longevity and increased morbidity due to a variety of disorders such as type 2 diabetes mellitus, cardiovascular diseases, hypertension, and hyperlipidemia[1]. The basis of the relation between obesity and the development of these disorders is still unclear. Recent data show enhanced circulating levels of inflammatory mediators in obese individuals[2,3]. These observations are interesting in the context of a suggested pathophysiological role for inflammatory mediators (such as TNFα) in the development of the obesity-related morbidity such as insulin resistance and cardiovascular disease[4]. Moreover, *in vivo* and *in vitro* studies have indicated an involvement of macrophage inhibitory factor (MIF) in the pathophysiology of insulin resistance[5].

In a previous study, we demonstrated a correlation between levels of inflammatory mediators, acute-phase proteins, and body weight[6]. Although a causative relation has not been found yet, it is suggested that elevated levels of C-reactive protein (CRP) are prognostic for the development of cardiovascular disease[7,8]. Also, other proteins related to inflammation are suggested to be associated with the development of obesity-related diseases such as plasminogen activator inhibitor-1 (PAI-1) and MIF[9,10]. Increased plasma levels of inflammatory markers and acute-phase proteins were present without physical evidence of acute or chronic inflammation in morbidly obese[6]. It is tempting to speculate that the metabolic stress caused by morbid obesity is responsible for the acute-phase response seen in these patients.

Several studies demonstrated that weight loss leads to reduced obesity-related comorbidity[11–13]. Based on the above-mentioned findings, we hypothesized that weight loss, after gastric restrictive surgery, resulting in a reduction of the metabolic stress, leads to a decrease in inflammatory mediators and acute-phase proteins in morbidly obese subjects.

Subjects and methods

A total of 27 consecutive subjects, which were admitted to the Surgical Department of the University Hospital Maastricht for surgical treatment of morbid obesity, were included in the study. The majority of these patients (n=26) underwent a primary operation for morbid obesity (vertical banded gastroplasty (VBG, n=11) or Lap-Band (n=15)). In one patient a gastric bypass was performed because of weight regain as a result of a staple line disruption after initial VBG. All subjects were otherwise healthy according to history, clinical examination, and routine laboratory findings. In particular, none of the studied subjects showed evidence of acute or chronic inflammatory disease.

Characteristics of the subjects are presented in Table 5.1. Weight loss was expressed as percentage excess weight loss (EWL), which could be calculated with the following formula: (preoperative weight - weight after reduction)/ (preoperative weight - ideal weight) * 100%.
Blood samples were collected after at least 8 h fasting, using evacuated blood collection tubes containing EDTA, at the day of admission to the hospital; 1 d before surgery; and 3, 6, 12, and 24 months after surgery. The blood samples were immediately put on melting ice and plasma was prepared by centrifugation at 1,400*g* for 10 min at 4°C. The plasma was centrifuged at 2,700*g* for 10 min at 4°C and stored in aliquots at -80°C. All participants gave written informed consent. The study was approved by the ethical committee of the University Hospital Maastricht.

Table 5.1 Characteristics of the study group.

Variable	Study group (n=27)
Age (yr)[a]	38.2 ± 7.5
BMI (kg/m^2)[a]	46.7 ± 5.8
Sex (male; female)	5; 22

[a] Mean ± SD.

VBG

In our hospital the procedure was performed as initially described by Mason[14]. Briefly, a small pouch of the stomach (approximately 15-20 ml) was created with a four-row linear stapler (TA-90B, United States Surgical Corp., Norwalk, CT) precisely to the angle of His, and a Dacron band of 5.0 cm in circumference placed through the window formed by a circular stapler (Premium Plus CEEA 31 mm, United States Surgical Corp.), leaving a very small opening for food to pass from the small pouch to the remaining stomach. Because of the small capacity of the gastric pouch, the amount of ingested food is considerably limited, leading to extensive weight loss[15].

Laparoscopic gastric banding (Lap-Band)

The Lap-Band (INAMED, Carpinteria, CA) is a new surgical technique to reduce body weight. The Lap-Band is made of soft silicone and equipped with an elastic balloon that can be inflated to the desired volume by means of injection postoperatively. After inflation the outlet diameter will be reduced, leading to diminished food intake and consequently to weight loss. This procedure was initially performed as described by Belachew et al.[16]. In short,

the Lap-Band was placed laparoscopically around the stomach. For definitive positioning of the band, a 15-ml calibration balloon advanced by the anesthetist and pulled up to the gastroesophageal junction was placed right below the cardia. Under this balloon the band was closed, and with three or four sutures, the ventral aspect of the greater curvature of the stomach is fixed to the pouch to ensure a stable anterior position of the band. Six weeks postoperatively the Lap-Band was insufflated when weight loss was insufficient (less than 6 kg). During the study period, the Lap-Band was insufflated as often as needed, up to a maximum of 4.5 ml, to induce sufficient weight loss (approximately 1 kg weight loss per week).

Reagents and materials

Monoclonal antibodies (mAbs) specifically directed against soluble TNFα receptor 55 (TNFR55) and soluble TNFα receptor 75 (TNFR75) were obtained as described elsewhere[17]. Polyclonal rabbit antisera anti-TNFR55 and anti-TNFR75 were obtained by immunizing rabbits with TNFR55 and TNFR75, respectively. Both mAbs 4G1 and 4F8 to leptin, were kindly provided by Dr. R. Devos (Hoffmann La-Roche, Welwyn Garden City, UK).
Human recombinant lipopolysaccharide binding protein (LBP), used as standard, was produced by transfected Chinese hamster ovary cells, kindly provided by Dr. P. Tobias (Research Institute of Scripps Clinic, La Jolla, CA). Polyclonal antibodies to human LBP were obtained by immunizing rabbits with human LBP. Human CRP was obtained from Dade Behring (Deerfield, IL); rabbit antihuman CRP and rabbit antihuman CRP-horseradish peroxidase were purchased from Dako (Glostrup, Denmark).
Human α1 acid glycoprotein (AGP) was obtained from Sigma (St. Louis, MO) and rabbit antihuman AGP from Dako. BSA was purchased from Sigma. Recombinant human leptin and MIF, a mAb antihuman MIF and a polyclonal antibody against human MIF were purchased from R&D Systems (Minneapolis, MN). Peroxidase-conjugated streptavidin was purchased from Dakopatts (Glostrup, Denmark) and 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Immunomaxisorp plates (Nunc, Roskilde, Denmark) were used for ELISAs.

Immunoassays

Plasma concentrations of both soluble TNFα receptors, leptin, LBP, CRP, AGP, MIF, and PAI-1 concentrations were measured using sandwich ELISAs. TNFR55, TNFR75, LBP, CRP, and AGP were quantified as described elsewhere[6,17,18]. The ELISAs for TNFR55, TNFR75, and LBP had a detection limit of approximately 100 pg/ml. The detection limit for CRP and AGP was approximately 10 pg/ml. The detection of plasma leptin levels was described

elsewhere[19]. In short, 96-wells plates were coated overnight at 4°C with an antihuman leptin antibody and diluted plasma samples as well as a dilution series of recombinant human leptin were added to the plate. Bound leptin was detected with a second antihuman leptin antibody, followed by peroxidase-conjugated goat antimurine IgA and TMB. The detection limit of this leptin assay is 0.04 ng/ml.
PAI-1 concentrations were measured using an ELISA, kindly provided by Dr. T. Kooistra (Leiden University Medical Center, Leiden, The Netherlands). In short, microtiter strip plates were coated with a highaffinity mAb PAI-1 3–3B against PAI-1. Detection for PAI-1 was carried out using a horseradish peroxidase-labeled antibody rabbit anti-PAI-1 followed by TMB. For MIF quantification, plates were coated with a mAb against human MIF. After adding the samples, detection was carried out with a biotinylated mAb against human MIF, followed by peroxidase-conjugated streptavidin and substrate.
All plasma samples were analyzed in the same run. When plasma concentrations exceeded the upper detection limit of the assay, samples were additionally diluted and analyzed in a separate run with an overlap to correct for interassay variation. The intra- and interassay coefficients of variance of the various assays were less than 10%.

Statistical analysis

Data are given as mean and SD except for MIF and PAI-1 because of no normal distribution. Statistical analysis was performed nonparametrically and two sided. The Wilcoxon signed ranks test was used to analyze differences between preoperative and postoperative values, within the morbidly obese subjects.
Pearson correlation coefficients were computed between body mass index (BMI) and leptin on the different time points. Statistical analyses were performed using the SPSS 10.0.7 statistical package (SPSS Inc.,Chicago, IL). $P<0.05$ was denoted as statistically significant.

Results

The effect of weight loss on plasma leptin and MIF

Table 5.1 summarizes the characteristics of the total of 27 morbidly obese patients studied. No differences in preoperative BMI was observed between both types of operation technique. As shown in Figure 5.1A, BMI significantly decreased after surgery from 46.7±5.8 kg/m^2 (mean±SD) preoperatively to 33.0±4.8 kg/m^2 at 24 months postoperatively ($P<0.001$). BMI decreased most

strikingly in the first 3 months postoperatively. As shown in Table 5.2, the relative EWL also was more in the period 0–3 months, compared with 4–6 months postoperatively. In the following 7- to 12-month period, the increase of EWL was significantly lower in comparison with the 0- to 6-month period. From 12 to 24 months, postoperative EWL hardly changed.

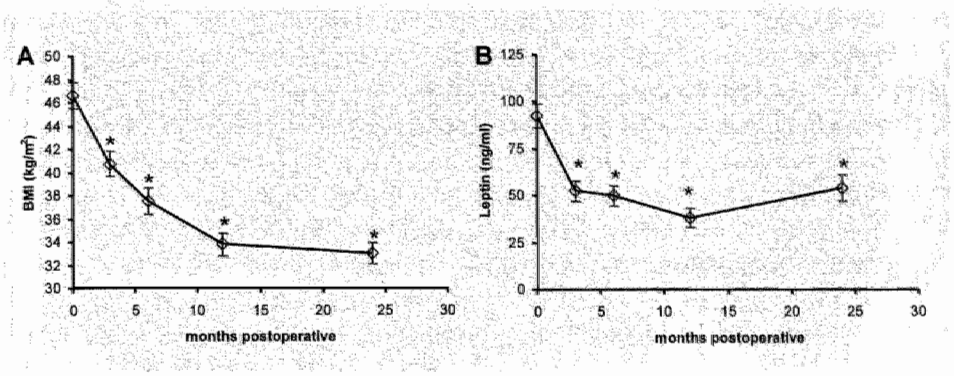

Figure 5.1 Effect of bariatric surgery on BMI and plasma leptin concentration.
BMI (A) and plasma leptin concentrations (B) were determined in 27 morbidly obese subjects preoperative as well as after gastric restrictive surgery. BMI and leptin both significantly decreased after gastric restrictive surgery. Values are shown as mean ± SD. *, significantly changed, compared with preoperative values.

Table 5.2 The effect of gastric restrictive surgery on EWL.

Time point	Total EWL (%)	Relative EWL (%)[a]
3 months postoperative (n=25)	28.3 ± 12.6[g]	28.3 ± 12.6[b]
6 months postoperative (n=26)	42.3 ± 16.2[g]	18.9 ± 9.6[c,f]
12 months postoperative (n=23)	60.3 ± 16.2[g]	31.8 ± 21.3[d]
24 months postoperative (n=27)	60.4 ± 18.5[g]	-9.4 ± 71.4[e]

[a] Relative EWL is EWL relative to the weight reached on the previous time point; [b] compared to preoperative situation; [c] compared to 3 months postoperative; [d] compared to 6 months post-operative; [e] compared to 12 months postoperative; [f] $P<0.001$ compared to relative EWL at 3 months postoperative; [g] $P<0.001$ compared to preoperative.

In a previous study, we demonstrated a correlation between leptin and body weight[6]. To evaluate the effect of weight loss on leptin levels in morbidly obese subjects, leptin was measured. As expected, plasma leptin concentrations decreased significantly postoperatively. Leptin levels decreased from 92.5±31.8 to 52.5±27.3 ng/ml at 3 months, 49.8±28.0 ng/ml at 6 months, and 38.0±25.8 ng/ml at 12 months and slightly increased to 53.9±35.7 ng/ml at 24 months postoperatively. As shown in Figure 5.1B, the reduction in leptin levels

was most pronounced in the first 3 months, in which the strongest decrease in EWL was observed.

In our previous study, we also demonstrated a correlation between body weight and acute-phase proteins. The acute-phase proteins MIF and PAI-1 are, besides leptin, also produced by adipose tissue[20–22]. Next to this, MIF is considered to be related to insulin resistance[5] as well as to the development of atherosclerosis[9]. PAI-1 is demonstrated to contribute to the increased susceptibility to atherogenesis, described in insulin-resistant patients with obesity[23,24]. In this context MIF and PAI-1 levels were measured in weight-losing morbidly obese subjects. Interestingly, in contrast to leptin levels, plasma MIF levels remained stable for the first 6 months postoperatively at a relatively low level. Thereafter MIF levels significantly increased to reach levels of lean individuals of 0.71±0.58 ng/ml at 24 months (Figure 5.2A). PAI-1 levels significantly decreased rapidly after surgery from 23.0±14.0 ng/ml preoperatively to 7.5±4.5 ng/ml at 12 months postoperatively (Figure 5.2B). PAI-1 levels displayed an initial strong reduction, being restricted to the first 3 months postoperatively. After 12 months PAI-1 levels remained rather stable, although with a wide SD, at 9.2±15.6 ng/ml up to 24 months postoperatively.

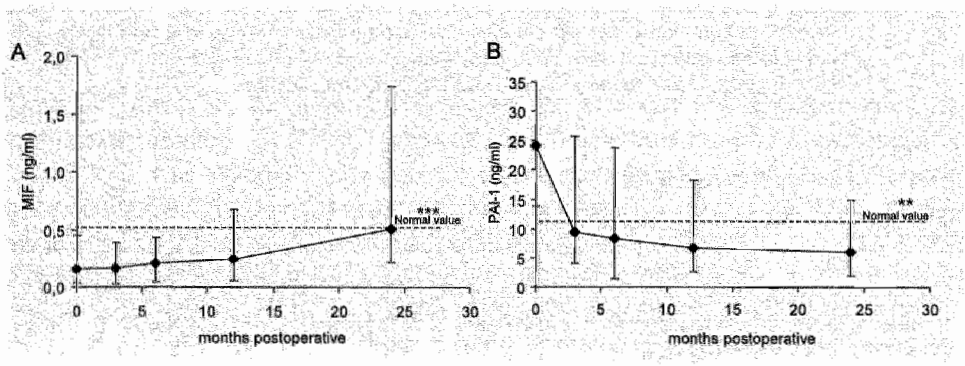

Figure 5.2 Effect of weight loss on MIF and PAI-1 levels. The effect of weight loss due to gastric restrictive surgery on plasma levels of MIF (A) and PAI-1 (B). Data are depicted as median ± interquartile ranges. *, Significantly changed compared with preoperative values. **, Mean of normal values according to Sasaki et al.[52]. ***, Median of normal values according to Lehmann et al.[53].

The effect of weight loss on LBP, CRP and AGP

In addition, the plasma levels of the acute-phase proteins LBP, CRP, and AGP were measured (Figure 5.3). LBP levels did not change significantly during the first 6 months postoperatively.

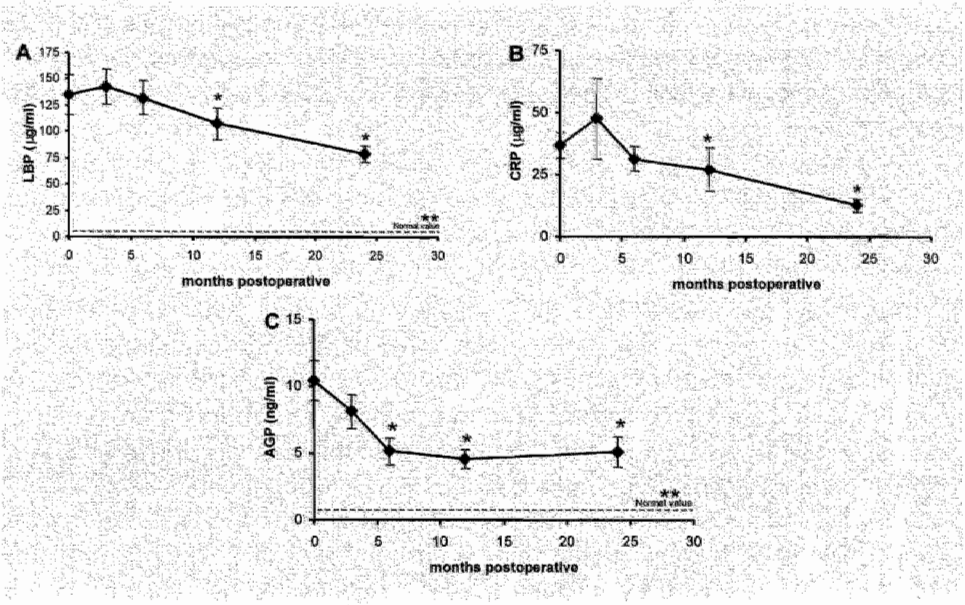

Figure 5.3 Effect of weight loss on acute-phase proteins.
The effect of weight loss due to gastric restrictive surgery on plasma levels of the acute phase proteins LBP (A), CRP (B), and AGP (C). Data are depicted as mean ±SD. *, Significantly changed, compared with preoperative values. **, Mean of normal values according to Prucha et al.[54]. ***, Mean of normal values according to van Dielen et al.[6].

However, at 12 and 24 months postoperatively, LBP levels decreased significantly (P<0.05; 107.6±77.4 and 78.9±39.4 µg/ml, respectively), compared with preoperative values (134.7±98.2). Similarly, CRP levels did not change up to 6 months postoperatively. Only after an EWL of more than 40%, a significant reduction in CRP levels could be observed (P<0.05).
Interestingly, the patterns of the three type I acute-phase protein levels differed. Both LBP and CRP showed a significant and a substantial reduction from 6 months after surgery onward. After 3 months both CRP and LBP levels were enhanced, although not significantly. In contrast, AGP levels displayed a similar pattern to that of PAI-1 levels. The AGP levels were decreased at 3 months after the operation. A significant decrease of 49% was reached at 6 months

postoperatively ($P<0.05$), compared with preoperative data, whereas from 6 months after surgery onward, AGP levels remained unchanged.

The effect of weight loss on both soluble TNFα receptors

Both soluble TNFα receptor levels did not change duringthe first year postoperatively. However, after 24 months TNFR55 was significantly decreased ($P<0.05$), compared with preoperative values, whereas TNFR75 remained unchanged. The levels of both soluble TNFα receptors at 24 months postoperatively were still significantly higher, compared with levels in lean subjects[6].

Discussion

In this study we investigated the influence of weight loss after surgical treatment of morbid obesity on the levels of inflammatory mediators. Gastric restrictive surgery led to a significantly decreased BMI. In the first 6 months after the operation, the BMI decreased substantially and reached a plateau at approximately 12 months. At this time point, the BMI was still above 30 kg/m^2 (the cut-off point of obesity). These data are in line with data of Naslund et al.[25] showing that after VBG the patients were still obese (BMI decreased from 44.4 preoperative to 32.6 kg/m^2 4 year after surgery). Also leptin, which is primarily expressed and released by adipose tissue[26], significantly decreased. On the different time points after gastric restrictive surgery, BMI and leptin were significantly correlated with each other (data not shown), implicating that, in general, leptin is a reliable indicator of fat mass.

Previously it has been demonstrated that inflammatory mediators are elevated in obese subjects[6,27,28]. In recent studies a regulatory role for leptin on the immune response has been proposed[29–32]. In this context, the elevated plasma leptin concentrations in morbidly obese patients may modulate the immunological homeostasis, leading to increased concentrations of acute-phase proteins and other inflammatory mediators, characteristic for a chronic inflammatory state. However, Hukshorn et al.[33] demonstrated that injections with high concentrations of recombinant leptin, in obese patients with already elevated plasma leptin levels, did not affect plasma levels of different acute-phase proteins, indicating that in obese individuals leptin does not directly regulate acute-phase protein levels.

Another important protein produced by adipose tissue is MIF, an acute-phase protein that is shown in animal models to be released by anterior pituitary cells in response to endotoxin[34]. Recent animal and *in vitro* studies suggested that MIF is linked to the development of atherosclerosis[9,10]. These findings might

suggest that high MIF concentrations are to be expected in morbidly obese subjects. In contrast, here it is demonstrated that MIF levels in morbidly obese subjects are low, although still in the normal range, and increase postoperatively with decreasing body weight. An explanation for these decreased MIF levels in morbidly obese subjects appears to be the reduced insulin sensitivity, often seen in morbidly obese subjects. The intracellular glucose level was found to be critical for the MIF protein content in adipose tissue.

In this context MIF mRNA expression of both epididymal fat pads of Tokushima fatty rats and Wistar fatty rats was found to be down-regulated, whereas plasma MIF levels of Wistar fatty rats increased upon treatment with pioglitazone, an insulin sensitizer[5]. These data suggest that the interplay between glucose and insulin is central to the regulation of MIF concentrations, leading to an increase of plasma MIF levels with enhanced insulin sensitivity. Because it is known that, with an increase in body weight, insulin sensitivity decreases[35], these data might explain the unexpected low concentrations of MIF measured in morbidly obese subjects.

Furthermore, MIF is demonstrated to sustain macrophage survival and function by suppressing activation-induced, p53-dependent apoptosis[36]. This finding combined with the preoperative low concentrations of MIF found in this report suggest an impaired macrophage function in morbidly obese subjects. If so, this might be an explanation for the observation that morbidly obese patients demonstrate more postoperative complications, compared with lean subjects[37,38]. However, further studies are necessary to unravel the pathophysiological role of MIF in morbidly obese subjects.

Although mainly produced in the stromal cell, besides leptin and MIF, PAI-1 is also adipose tissue derived[36]. Mitchell et al.[36] demonstrated that PAI-1 expression was 5-fold higher in the visceral fat that in the sc fat. Next to this, Janssen and Ross[39] demonstrated that during weight loss the reduction in visceral fat was faster than in sc fat. In line with these data, the present results show that PAI-1 levels decreased rapidly with a decrease in body weight.

On the other hand, despite significant weight loss and concurrent decrease of plasma leptin levels, the levels of the acute-phase proteins LBP and CRP and both TNFα receptors remained elevated up to 6 months or longer. The acute-phase protein AGP, like PAI-1, showed a rapid decrease after surgery. Two years after gastric restrictive surgery, all acutephase proteins were significantly reduced.

Different explanations can be proposed for the initially sustained elevation of inflammatory mediators. First, the effect of the operation and the subsequent healing process might be a possible explanation for the enhanced inflammatory state during the first 6 months postoperatively. However, various studies demonstrate that the highest CRP levels occur 12–48 hour after surgery[40–42]

and will remain elevated only for a period of maximum 12 days post-operatively[42]. Second, a nonalcoholic steatohepatitis might be an explanation for the sustained elevation of inflammatory mediators. Rapid weight loss can result in a mild increase in inflammatory lesions (hepatitis)[43]. Increased concentration of intracellular fatty acids, as has been observed during rapid weight loss, could explain these inflammatory lesions in the liver after weight loss. Such elevated levels of free fatty acids may be directly toxic for the liver or lead to oxidative stress. However, severe nonalcoholic steatohepatitis and hepatic failure are seldom described after gastroplasty or gastric bypass[44].

A third possible explanation for the prolonged elevation of inflammatory mediators might be an enhanced metabolic stress response due to relative starvation. It is demonstrated in very malnourished anorexia nervosa patients that TNFα and IL-1β were elevated, compared with healthy controls. After refeeding these inflammatory mediators returned to normal levels[19,45–47]. If we assume that extensive weight loss after gastric restrictive surgery is comparable with starvation, this could be an explanation for the prolonged elevation of inflammatory mediators. We consider therefore that from the moment onward that the body weight is stabilized at approximately 12 months postoperatively, the metabolic instability will improve, leading to a reduction in inflammatory mediators. This was reflected by the reduced CRP levels at 24 months after gastric restrictive surgery. Taken together, CRP levels were postoperatively not directly correlated with BMI but rather also influenced by the disturbed metabolism.

Next to the acute-phase proteins, soluble TNFR55 and TNFR75 were measured. In earlier reports, levels of soluble TNFα receptors were demonstrated to be of value in characterizing an inflammatory response[48,49]. In an earlier report we demonstrated an increase in both soluble TNFα receptors with increasing body weight[6]. Whether this elevation of soluble TNFα receptor levels is caused by elevated levels of TNFα, produced by adipocytes, is as yet unknown. If so, soluble TNFα receptor levels would decrease with decreasing body weight due to a reduction in body fat. However, in this report we demonstrate a sustained elevation, compared with healthy controls, up to one year postoperatively for both soluble TNFα receptors, despite the weight loss. The fact that the studied subjects were still obese (mean BMI was 33.0 kg/m^2), even at 24 months after gastric restrictive surgery, might be an explanation for the sustained elevation of both soluble TNFα receptors.

An increasing number of studies demonstrate a central role for inflammatory processes in the pathogenesis of cardiovascular disease and insulin resistance[8,50]. Plasma levels of several markers of inflammation have been found to be associated with an increased cardiovascular risk in a variety of clinical settings. Especially CRP and PAI-1 are important prognostic factors for the development of cardiovascular disease[24,51]. As demonstrated in this report,

during weight loss due to gastric restrictive surgery, almost all inflammatory mediators measured eventually decrease. Next to this, it is demonstrated by others that after gastric restrictive surgery, obesity-related comorbidities like cardiovascular disease and diabetes mellitus are strongly reduced[12,13].
So hypothetically the decreased inflammatory mediators after weight loss in morbidly obese patients, as shown in this study, may be related to an improvement of comorbidities in these patients.

In summary, this study demonstrates that after gastric restrictive surgery, BMI and leptin levels significantly decrease, whereas MIF levels increase. Despite the extensive weight loss, both soluble TNF receptors as well as the acute phase proteins LBP and CRP remained elevated up to six months postoperatively, suggesting an ongoing inflammatory state in these obese subjects. However, two years after gastric restrictive surgery, when the body weight has stabilized, the levels of all inflammatory mediators were strongly decreased.
Hypothetically this improved metabolic state might be an explanation for the reduced obesity-related comorbidity after successful surgical treatment for morbid obesity.

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Chapter 6

Leptin and soluble leptin receptor levels in obese and weight-losing individuals

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Abstract

Background
To investigate soluble leptin receptor (sLR) in plasma, specific anti-sLR monoclonal antibodies were developed. Western blot analysis and size exclusion fractionation demonstrated sLR in plasma with a molecular mass of approximately 180,000. Next to this, the presence of sLR-leptin complexes in plasma was confirmed.

Methods
Using the developed monoclonal antibodies, a specific sLR ELISA was developed, which measured in plasma both free and sLR bound to leptin. sLR appeared to inhibit leptin concentrations measured in four different leptin assays indicating that these assays primarily measure free leptin and underestimate the total leptin present in plasma.

Results
Furthermore, plasma levels of sLR and leptin were measured in 21 lean individuals and in 30 morbidly obese subjects before and 3, 6, and 12 months after gastric restrictive surgery. Preoperatively, leptin concentrations significantly correlated with body mass index ($r=0.796$, $P<0.001$). In contrast, sLR significantly inversely correlated with body mass index ($r=0.294$, $P<0.05$). In lean subjects, the molar ratio of free leptin to sLR was 1:1, whereas in morbidly obese subjects a ratio of 25:1 was found. After weight loss due to surgery, leptin levels rapidly decreased and sLR levels slowly increased to reach normal values at 12 months postoperatively.

Conclusions
We conclude that sLR levels are significantly decreased, whereas leptin levels are significantly increased in morbidly obese subjects compared with lean individuals.

Introduction

Leptin, a cytokine that is primarily expressed by adipose tissue[1], is considered to be involved in satiety regulation in mice[2,3]. Leptin controls food intake by its interaction with the leptin receptor in the brain[4,5]. As a consequence, leptin-deficient and leptin receptor-deficient mice show an obese phenotype[5–8]. Furthermore, administration of leptin to leptin-deficient or diet-induced obese mice resulted in a significant decrease of food intake[9,10].

The action of leptin is mediated by the leptin receptor, which belongs to the class I type cytokine receptor family[11–14]. In humans, four different mRNA splice variants of the leptin receptor have been identified, consistent with a membrane bound receptor with a long cytoplasmatic domain and three membrane bound receptors with a short cytoplasmatic domain. Furthermore, soluble leptin receptor is generated by proteolytic cleavage of membrane-anchored receptors, indicating that the leptin receptor might have other functions besides signal transduction[15]. In blood, leptin is suggested to circulate both in free form as well as bound to a soluble leptin receptor and possibly also to other as yet unidentified binding proteins[16]. Binding of leptin with soluble leptin receptor has been suggested to increase the bioavailability of leptin in plasma[16,17] as well as to decrease binding of leptin to membrane bound leptin receptors[18]. The role of the soluble leptin receptor in the regulation of the physiological function of leptin is hitherto unclear.

In contrast to obesity in leptin-deficient mice, in man obesity is associated with increased leptin plasma concentrations[19]. Based on murine experiments, it has been postulated that adipose tissue signals via increasing leptin levels that food intake has been sufficient[20]. Assuming that a similar mechanism holds for man, lack of satiety in obese persons could indicate insensitivity to elevated leptin levels. Because circulating soluble leptin receptors have been reported to be involved in leptin function, these receptors could be involved in the satiety response.

In this study, we investigated circulating leptin receptor levels. To this end, an ELISA for the quantification of soluble leptin receptor (sLR) was developed and characterized. Using this assay, we studied circulating soluble leptin receptor and leptin in lean and obese subjects as well as in obese subjects during weight loss.

Patients and methods

Subjects

In total, 51 subjects were included in the study. The study population comprised thirty morbidly obese and 21 healthy subjects, matched for gender

and age. The morbidly obese patients were admitted to the Surgical Department of the University Hospital Maastricht for gastric restrictive surgery. Operated patients were evaluated in the outpatient clinic at 3, 6, and 12 months postoperatively. All subjects were otherwise healthy according to history, clinical examination, and routine laboratory findings. In particular, none of the studied subjects had evidence of acute or chronic inflammatory disease. Characteristics of the subjects are presented in Table 6.1.
Blood samples of lean subjects were taken after an overnight fast, using evacuated blood collection tubes containing EDTA.

Table 6.1 Characteristics of the study population.

Variable	Morbidly obese group (n=30)	Control group (n=21)
Age[a]	37.2 ± 8.2	33.2 ± 7.8
BMI[b] (kg/m^2)	46.1 ± 5.1	24.6 ± 3.1
Sex (male/female)	7 / 24	5 / 16

[a] Age is given as mean and SD; [b] BMI = body mass index, given as median and SD.

In the patient group, blood was taken before surgery or at the outpatient clinic 3, 6, and 12 months after operation. All preoperative blood samples were taken at 11:00 hours, during preoperative assessment the day before surgery, after an overnight fast. Postoperative samples were taken at approximately 15:00 hours, after minimally 7 hours fast. Blood samples were immediately put on melting ice, and plasma was prepared by centrifugation at 1,400*g* for 10 min at 4°C. The plasma was spun again at 2,700*g* for 10 min at 4°C and recovered plasma was stored in aliquots at -80°C. The study was approved by the ethical committee of the University Hospital Maastricht (Maastricht, The Netherlands). All subjects gave informed consent.

Reagents

BSA was purchased from Sigma (St. Louis, MO). Bovine calf serum purchased from HyClone Laboratories, Inc. (Logan, UT) was heated at 56°C for 30 min before storage at 4°C. Human recombinant leptin receptor/Fc chimera (sLR-Fc) as well as recombinant human leptin were purchased from R&D systems (Minneapolis, MN). Purification of this chimeric protein was performed using protein G-Sepharose 4 Fast Flow affinity chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Soluble human leptin receptor-myc tagged was kindly provided by Prof. dr. J. Tavernier (Ghent University, Ghent, Belgium).

Production and selection of monoclonal antibodies (mAb)

BALB/c mice (obtained from the central animal facilities of the University of Maastricht (Maastricht, The Netherlands)) were immunized by ip injections with human recombinant sLR-Fc, using Special Oil Phase (Specol) as adjuvant[21]. These immunizations were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Maastricht. Spleen cells of the immunized mice were fused with SP2O mouse myeloma cells and plated in 96-well plates. Supernatants of wells containing proliferating cells were screened for the presence of anti-sLR Ab and antihuman IgG Ab (the latter to exclude antihuman IgG reactivity), using ELISA methodology. Hybridomas producing anti-sLR antibodies were subcloned. Isotypes of the mAb were determined using a mAb isotyping mouse test kit (HyCult Biotechnology bv, Uden, The Netherlands).
Anti-sLR mAb were purified from culture supernatant by affinity chromatography on a protein G-Sepharose 4 fast flow column (Amersham Pharmacia Biotech). Antibodies were biotinylated using biotin-X-NHS (Calbiochem, La Jolla, CA).

Soluble leptin receptor sandwich-ELISA

The four generated anti-sLR mAb were tested for their usefulness to construct a sandwich ELISA assay for sLR. Based on pilot experiments, the following method to measure sLR was developed and evaluated. sLR specific mAb 2F1 was coated at a concentration of 3 μg/ml in PBS overnight at 4°C onto 96-well plates (Immuno-Maxisorp; Nunc, Roskilde, Denmark). Free sites were blocked by one hour of incubation with 1% BSA in PBS at room temperature. Wash buffer consisted of distilled water containing 0.1% Tween 20 and samples were diluted in PBS with 0.1% BSA.
Plates were washed four times after each incubation step. Test samples as well as human recombinant sLR-Fc, used as standard, were incubated for one hour at room temperature. In this experiment, biotinylated mAb 4C3 was used as detection antibody for one hour at room temperature. HRP-streptavidin conjugate (Zymed Laboratories, Inc., San Francisco, CA) was used to develop the color reaction in combination with 3, 3', 5, 5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and H_2SO_4. Color intensity was measured by determination of the absorbance at 450 nm using a micro-ELISA reader.

Leptin ELISA

For detection of plasma leptin levels, plates were coated overnight at 4°C with the murine antihuman leptin mAb 4G1. After blocking with 1% BSA in PBS,

diluted plasma samples and a dilution series of recombinant human leptin, the standard curve, were added to the plate. Bound leptin was detected by incubation with antihuman leptin mAb 4F8, followed by peroxidase-conjugated goat antimurine IgA. 3, 3', 5, 5'-tetramethylbenzidine was used as substrate for peroxidase, and color intensity was determined by measuring absorbency at 450 nm. The detection limit of this leptin assay is 0.04 ng/ml. Both mAb, 4G1 and 4F8, were kindly provided by Dr. R. Devos (Hoffmann La-Roche, Welwyn Garden City, UK). Leptin was also measured using two leptin ELISAs (high sensitive and normal leptin kit, BioVendor, Brno, Czech Republic), with a detection limit of 0.08 and 0.2 ng/ml respectively. In addition, a leptin RIA was used (Linco Inc., St. Charles, MO) with a detection limit of 0.5 ng/ml.

Purification of native soluble leptin receptor from plasma

To purify native sLR in a single step by affinity chromatography, the murine antihuman leptin receptor mAb 2F1 was coupled to CNBr-Sepharose beads (2 mg/ml gel; Amersham, Pharmacia Biotech). Human plasma with a high sLR concentration (>400 ng/ml) was passed over the affinity column at room temperature. After extensive washing, proteins bound to the column were eluted with 0.1 m glycine-HCl buffer, pH 2.5. The quantity of the eluted natural sLR was estimated by ELISA.

Determination of leptin binding properties of sLR present in plasma

To study the binding-capacity of sLR for leptin, a study with ^{125}I-leptin (Linco Inc.) was performed. sLR-Fc, recombinant sLR-myc tagged or plasma derived sLR, were incubated overnight with different concentrations recombinant leptin, followed by overnight incubation with ^{125}I-leptin. The next day, samples were added to an ELISA plate, coated with mAb 2F1 anti-sLR, incubated for 3 hours, followed by five washings with distilled water containing 0.1% Tween 20. After washing, bound ^{125}I-leptin was eluted with glycine-HCl pH 2.5, for 10 min. and radioactivity in the supernatant was measured using a universal gammacounter (type 1282 compugamma CS, LKB Wallac, Inc., Turku, Finland). All incubation steps were performed at 4°C.

Furthermore, 2 mg pegylated leptin (Leptin-PEG, kindly provided by Dr. W. Saris and Dr. C. Hukshorn, University of Maastricht) was coupled to CNBr-Sepharose beads (Amerdsham Pharmacia Biotech). Plasma was incubated overnight at 4°C with leptin-PEG Sepharose. After removal of the Sepharose beads by centrifugation (500 rpm) during 5 min., leptin and sLR-levels were measured by ELISA in the supernatant.

After extensive washing, the beads were transferred to Laemmli buffer, heated, and subjected to SDS-PAGE electrophoresis and Western blotting for leptin receptor and leptin.

Size exclusion fractionation of plasma

To determine the distribution of sLR and leptin in plasma, a plasma sample (2 ml) was fractionated at 4°C, using Sephacryl S-300 HR gel filtration. Fractions of 3 ml were automatically collected with a fraction collector (type 2128, Bio-Rad Laboratories, Inc., Hercules, CA). sLR and leptin concentrations in the fractions were measured with the described sLR and leptin assay. Furthermore, human IgG and human albumin were quantified by in house assays and human Liver fatty acid binding protein (l-FABP), a 14 kDa protein, by a commercial ELISA (Hbt, Uden, The Netherlands) to determine the fractions with the highest concentration of these proteins with known molecular mass.

Western blotting for soluble leptin receptor and leptin

For SDS-PAGE electrophoresis, sLR-Fc and plasma derived sLR were heated for 5 min at 95°C in Laemmli buffer and electrophoresed in a polyacrylamide/SDS gel. Proteins were transferred to nitrocellulose membranes, which were subsequently blocked with 0.1 m Tris-buffered saline 0.1% Tween 20 and 5% non-fat dry milk for one hour at room temperature. Next, membranes were incubated with biotinylated murine antibodies or murine antibodies to human sLR or leptin respectively, in Tris-buffered saline 0.5% nonfat dry milk for one hour. After washing, the membranes were incubated with streptavidin peroxidase or peroxidase conjugated goat antimouse antibodies. Positive bands were detected using the chemiluminescent substrate Supersignal West Pico (Pierce Chemical Co., Rockford, IL) and were transferred onto a X-ray film.

Statistical analysis

The Mann-Whitney U test was used to analyze differences between groups. The Wilcoxon signed ranks test was used to analyze differences between preoperative and postoperative values, within the morbidly obese subjects.
Pearson correlation coefficients were computed between body mass index (BMI), sLR, and leptin for the complete group of morbidly obese subjects preoperatively and lean controls. Statistical analyses were performed using the SPSS 10.0.5 statistical package (SSPS Inc., Chicago, IL). $P<0.05$ was denoted as statistically significant.

Results

Generation of anti-sLR mAb and development of a specific sLR-assay

Four positive anti-sLR mAb secreting hybridomas (all IgG1), which detected solid phase sLR-Fc chimera in ELISA, were further characterized by Western blotting. Two mAb (2F1 and 4C3) recognized recombinant sLR-Fc, sLR-myc tagged and natural derived plasma sLR, purified using antisLR Sepharose (Figure 6.1). The antibodies reacted with plasma derived sLR with two bands: one band at approximately 180 kDa and one weak band at approximately 90 kDa. Both antibodies also reacted with recombinant sLR-myc tagged at approximately 90 kDa, which is in line with data of Lewandowski et al.[20], who reported similar results for the very same recombinant sLR protein.

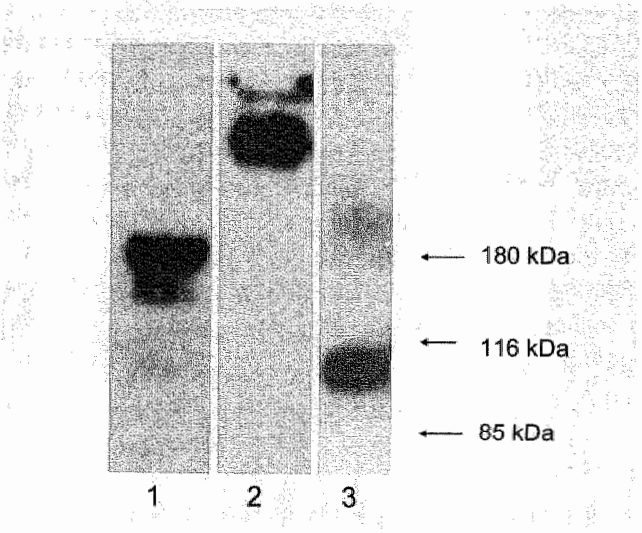

Figure 6.1 Western blot analysis of sLR. Western blot analysis of plasma derived soluble leptin receptor, purified using anti-sLR Sepharose (lane 1), unreduced sLR-Fc (lane 2) and recombinant sLR-myc tagged (lane 3). In plasma, sLR was detected primarily as a band of approximately 180 kDa, and a very weak band at approximately 90–100 kDa. sLR-myc tagged was detected at approximately 90–100 kDa. Unreduced sLR-Fc showed a thick band at approximately 380 kDa. In this experiment, mAb 4C3 was used for detection. Similar results were obtained with mAb 2F1 (data not shown).

Furthermore, both mAb reacted with unreduced sLR-Fc at bands of approximately 380 kDa (Figure 6.1). After reduction of sLR-Fc, mainly a band at approximately 180 kDa was found (data not shown).

Next, the usefulness of the antibodies to detect sLR in a sandwich ELISA was assessed. The combination of mAb 2F1 as catching antibody with biotinylated mAb 4C3 as detector was found to form a sLR-ELISA, with a lower detection limit of 1.5 ng/ml. To determine whether sLR-Fc and sLR present in plasma were detected by this ELISA with similar kinetics, dilution curves of plasma and sLR-Fc were assayed. As depicted in Figure 6.2A, similar dilution curves were obtained in the sLR assay indicating detection of sLR in plasma and sLR-Fc with similar kinetics. Next, we investigated whether the presence of leptin interfered with the detection of sLR. To this end, a concentration range of leptin was added to both sLR-Fc in PBS 0.1% BSA and plasma. The quantification of sLR was not affected by leptin added in excess (Figure 6.2B). The absence of effect of leptin addition on sLR quantification strongly suggests that the sLR assay measures not only free sLR, but also sLR-leptin complexes. Next, the influence of natural leptin, as present in plasma, on the measurement of native sLR was studied. To this end, plasma samples containing respectively higher or lower leptin levels were mixed and incubated for one hour with samples, containing respectively either low or high sLR levels. In these mixed samples, sLR levels were directly proportional to the dilution of the samples (Figure 6.2C). These data support the previous data and indicate that the presence of leptin in plasma does not influence quantification of sLR.

Characterization of sLR present in plasma

Earlier reports suggested the presence of sLR-leptin complexes in plasma[14,16,21]. In this context, we determined the distribution of sLR and leptin in plasma by size exclusion fractionation. Plasma (2 ml) was fractionated using Sephacryl S-300 HR gel filtration and sLR and leptin concentrations were measured by ELISA (Figure 6.3). Fractions 49–60 contained sLR with a peak at fraction 55. The sLR peak preceded the peak of human IgG (approximately 150 kDa) with a peak at fraction 56, corresponding to a molecular mass of approximately 160–180 kDa. These data are in line with size exclusion fractionation experiments of others[20] and the Western blot data as reported above. Size exclusion fractionation did not allow discrimination between putatively in plasma present sLR-leptin complexes (estimated MW 180+16 kDa) and free sLR.

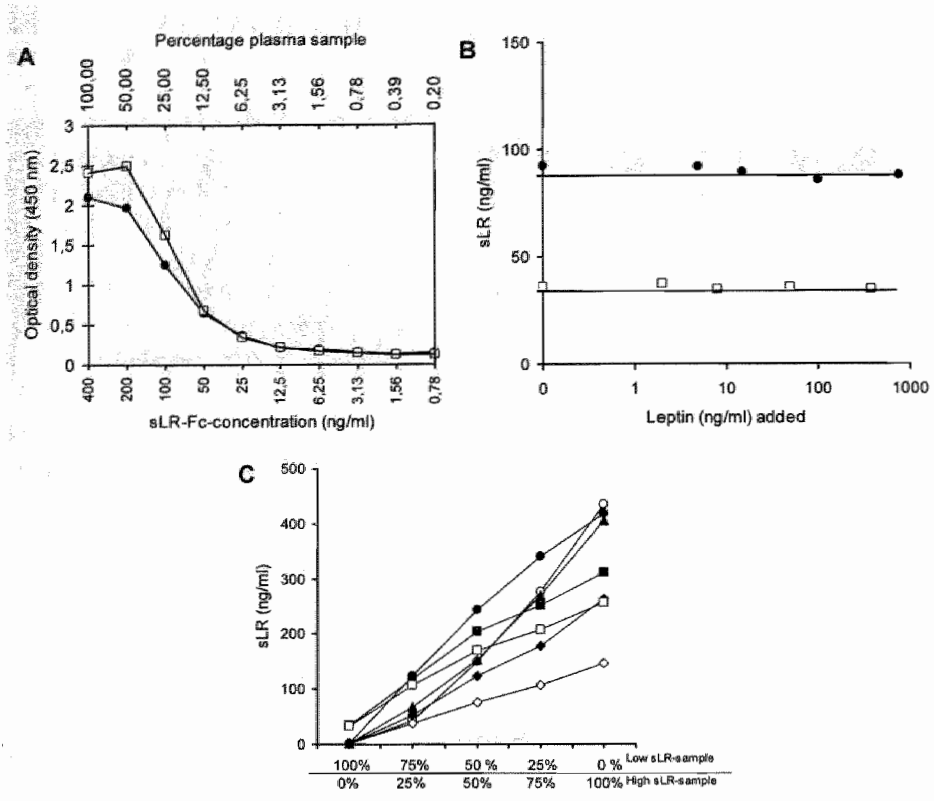

Figure 6.2 ELISA for quantification of soluble leptin receptor. A, Dilution-curves of plasma sample (□) and of sLR-Fc (●). Both curves show similar kinetics. B, Addition of leptin does not affect quantification of soluble leptin receptor concentration. A range of leptin concentrations was added to 100 ng/ml sLR-Fc in PBS + 0.1% BSA (●) and to plasma containing 35 ng/ml native sLR (□). C, Influence of leptin on the quantification of sLR was studied by making dilution curves of 7 mixtures of each two plasma samples with a high and low soluble leptin receptor level. Every point on the curves represents a mixture of the indicated ratio of dilution of both samples.

Leptin, as detected by the leptin ELISA, eluted in fractions 40–84 with the peak in fraction 75. The leptin peak preceded the LFABP-peak (approximately 14 kDa) with a peak at fraction 79. Furthermore we investigated whether the sLR molecules, detected in plasma have leptin binding capacity. To this end, plasma of a lean subject (BMI of 21.5 kg/m^2) was incubated overnight at 4°C with leptin Sepharose and both soluble leptin receptor and leptin were quantified in such treated plasma. A strong decrease of the sLR concentration from 115 ng/ml (~1.25 nm sLR) to undetectable levels (<1.5 ng/ml) was observed, whereas plasma incubated with antibodies to human albumin bound to Sepharose showed no change in sLR concentration (Table 6.2).

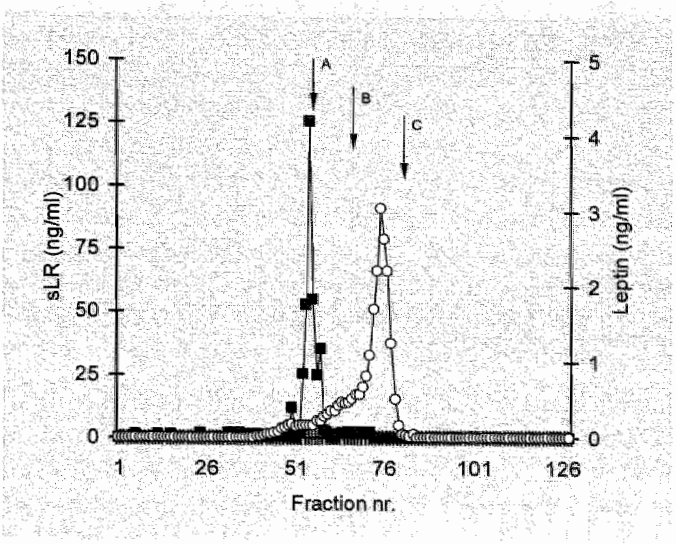

Figure 6.3 Size exclusion fractionation of plasma. Chromatographic pattern of plasma, following Sephacryl S300 chromatography. The two peaks correspond to sLR (■; *left peak*) and free leptin (o; *right peak*). The three *capital letters* correspond to the top peaks of human IgG (A; 150 kDa), human albumin (B; 60 kDa) and human L-FABP (C; 14 kDa). All fractions were assayed using human IgG, albumin, L-FABP, sLR, and leptin ELISAs.

Interestingly, the depletion of sLR was accompanied by an increase of the plasma leptin concentration. The leptin concentration raised from 21.9 to 34.9 ng/ml (~1.36 to 2.18 nm leptin), an increase of 0.82 nm leptin. In the control experiments, leptin concentration did not change in plasma incubated with antibodies to human albumin which were coupled to Sepharose. Furthermore, incubation of leptin-Sepharose with PBS showed only minimal leakage of the Sepharose-bound leptin. The plasma depletion of sLR by leptin-Sepharose implicates that the soluble leptin receptor, as detected by our ELISA, has leptin binding capacity. Moreover, the observed increase of leptin in such treated plasma strongly suggests dissociation of leptin-sLR complexes in the presence of an excess leptin bound to Sepharose. Presuming that all leptin-sLR complexes present in plasma are dissociated, and based on the assumption that leptin and sLR bind in a 1:1 ratio, these data might indicate that at least 64% of all sLR present in plasma was bound to leptin [increase in leptin in nm (0.82) divided by the concentration of sLR in nm (1.25)*100%].

Table 6.2 Leptin and sLR-concentrations after incubation with leptin sepharose beads or control sepharose beads[a].

Incubation	Leptin-concentration[b]	sLR-concentration[b]
Control-S[c] + plasma.	21.9	115.7
Control-S + PBS	0	0
Leptin-S[d] + plasma	34.9	0
Leptin-S + PBS	1.5	0

[a] Leptin and sLR were measured in human plasma using ELISA, after incubation for 24 hours of plasma with leptin-PEG Sepharose or control Sepharose.; [b] Data are given in nanograms per millilitres; [c] Control-S, mouse antirat MAC1 antibodies coupled to sepharose beads; [d] Leptin-S, leptin-PEG coupled to sepharose beads.

Next, experiments were performed to confirm that the sLR-receptors, detected by ELISA in plasma, are capable of binding leptin. To this end, sLR-Fc, recombinant sLR-myc tagged and plasma derived sLR were incubated with a concentration range of recombinant leptin for 24 hours, where after ^{125}I-leptin was added. Subsequently, this mixture was added to an ELISA plate, coated with the mAb 2F1 (anti-sLR). As depicted in Figure 6.4, ^{125}I-leptin binding with recombinant sLR or sLR-Fc decreased with increasing concentration of recombinant cold leptin, which implicates that leptin binds to sLRmyc tagged as well as to sLR-Fc. The data show that mAb 2F1, used as coating antibody in the newly developed sLR-assay, detects sLR capable of binding leptin.

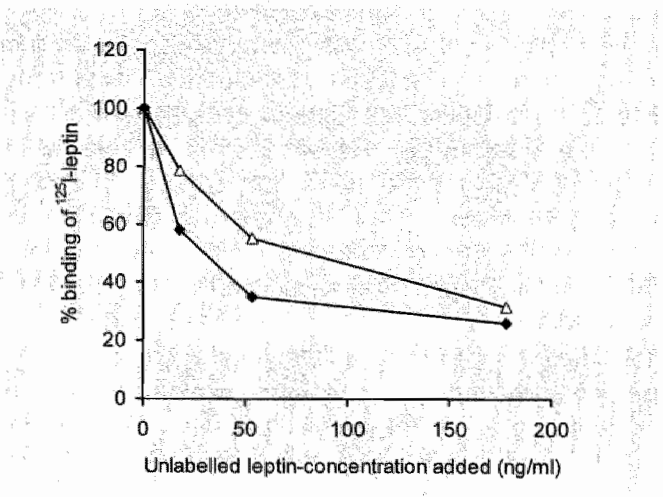

Figure 6.4 Binding studies of ^{125}I-leptin to sLR. Percentage binding of ^{125}I-leptin (~7000 cpm), added to 100 ng/ml sLR-Fc (♦) or recombinant sLR-myc tagged (Δ), after overnight preincubation at 4°C with different concentrations of cold leptin. Concentrations of cold leptin to sLR were in a molar ratio of respectively 0 to 1, 2:1, 6:1, and 20:1 for sLR-Fc and 0:1, 1:1, 3:1, and 10:1 for sLR-myc tagged. When ^{125}I-leptin was added to plasma derived sLR, no binding could be observed (data not shown).

However, no binding of ^{125}I-leptin with plasma derived sLR was found (data not shown). An explanation for the latter might be the high percentage of saturation of sLR in plasma with leptin, as our data above indicated. To ascertain the presence of sLR-leptin complexes in plasma, plasma was incubated with leptin Sepharose, Sepharose carrying antibodies to human leptin receptor or with control Sepharose. To diminish aspecific binding to Sepharose beads, plasma was three times 24 hours precleared with control Sepharose (Sepharose beads with mouse anti rat MAC1 antibodies). The proteins attached to the Sepharose beads were analyzed by Western blot. Figure 6.5 shows that anti-sLR Sepharose bound both leptin receptor and leptin, indicating a binding of mAb 2F1 with both free sLR and the sLR-leptin complex. Furthermore, incubation of plasma with leptin-Sepharose led to binding of sLR to the leptin-Sepharose beads, indicating that the mAb used for the sLR-ELISA are capable of detecting sLR with binding capacity for leptin as well as sLR-bound to leptin.

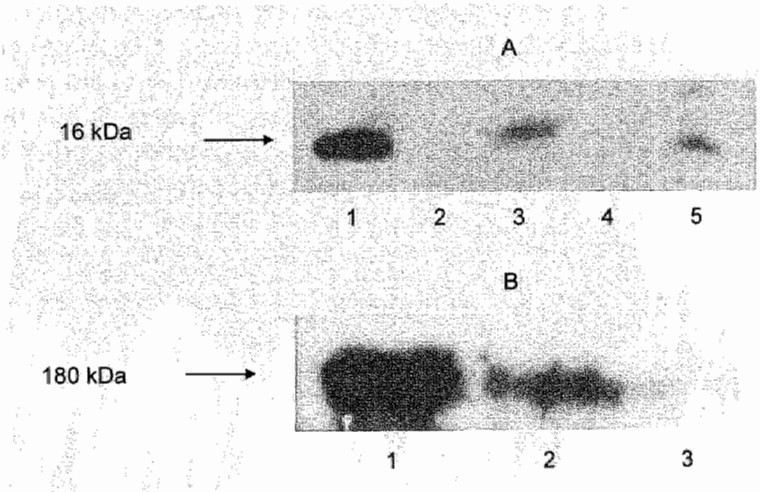

Figure 6.5 Western blot analysis of leptin and sLR. A, Western blot analysis on the presence of leptin in plasma, on Sepharose beads coated with anti-sLR antibody 2F1 and on control Sepharose beads. After overnight incubation with plasma and carefully washing, leptin was eluted from Sepharose beads coated with anti-sLR antibodies (lane 3), indicating the presence of sLR-leptin complex bound to the beads. As expected, after incubation with plasma, no leptin was present on Sepharose beads with anti rat MAC1 antibodies (lane 4), but still detectable in the plasma (lane 5). Recombinant leptin (lane 1) was used as positive control and PBS as a negative control (lane 2). In this experiment, mAb 4F8 was used for detection. B, Western blot analysis on the presence of sLR on Sepharose beads coated with leptin-PEG or coated with anti sLR antibodies. After incubation with plasma, sLR was eluted from Sepharose beads with anti sLR antibodies (lane 1) and from Sepharose beads with leptin-PEG (lane 2). PBS (lane 3) was used as a negative control. In this experiment, biotinylated mAb 4C3 was used for detection protection.

sLR inhibits detection of leptin by four different widely used leptin assays

The finding that plasma contains sLR, which appeared to be to a large extend bound to leptin, and the observation that leptin was not detectable in the gel filtration fractions that contained sLR, prompted us to investigate the capacity of the leptin assay used above, to detect leptin bound to sLR. In addition, other widely used leptin-assays were studied.
Recombinant human leptin (3 ng/ml ~0.2 nm) was incubated for 48 hours at 4°C with 0, 9, 18, and 90 ng/ml (~0, 0.1, 0.2 and 1.0 nm) leptin free sLR-Fc or with native sLR (with unknown leptin concentration), purified from plasma with an anti-sLR column.
As depicted in Table 6.3, the detection of leptin in all four leptin assays was reduced with increasing sLR-Fc as well as native sLR concentrations. There appears to be a difference between the assays in extend of inhibition with the Linco RIA assay being less consistently inhibited than the other assays. Furthermore, it appeared that the native sLR was more effective in inhibiting the leptin assays, than the sLR-Fc. A fact that might be due to the presence of the Fc-tail in the molecules. Taken together, these data indicate that the leptin assays investigated are strongly inhibited by the presence of sLR in the test sample and most likely by the formation of sLR-leptin complexes.

Table 6.3 Percentage inhibition of leptin detected in different leptin assays in presence of sLR[a].

Native sLR-concentration added (ng/ml)[b]	Linco Ria	2F1/4F8-assay[c]	BioVendor-assay	High sensitive BioVendor-assay
0				
9	80	90	74	44
18	79	98	100	100
90	ND[d]	97	100	63
sLR-Fc concentration added (ng/ml)[b]	**Linco Ria**	**2F1/4F8-assay**	**BioVendor-assay**	**High sensitive BioVendor-assay**
0				
9	51	26	51	-9
18	79	32	72	51
90	100	98	100	59

[a] Data given are percent inhibition of value obtained in absence of sLR. In absence of sLR, therefore by definition, 0% inhibition is found, which is not indicated; [b] The samples, containing 3 ng/ml leptin, were incubated 24 hours with both native sLR and sLR-Fc; [c] 2G1/4F8-assay is the leptin assay with mAb 2G1 and 4FB as catching and detection Ab, respectively; [d] Not done.

Next, the effect of sLR on the leptin concentration measured in plasma was also studied. To this end, plasma was incubated overnight with different concentrations of sLR-Fc. Leptin concentrations were measured using the

leptin-ELISA with mAb 4G1 and 4F8. Incubation with sLR-Fc strongly decreased leptin concentrations detected (data not shown). This decrease in detectable leptin concentration was in line with the above described data, indicating that also in plasma the assay cannot detect leptin present in sLR-leptin complexes.

BMI, leptin, and sLR concentrations in lean and morbidly obese subjects

Using the characterized assays for leptin and sLR, we measured these parameters in subjects with high and low BMI. Table 6.1 summarizes the characteristics of 30 morbidly obese subjects and 21 healthy subjects studied. The lean subjects had a mean BMI±SD of 24.6±3.1, and the morbidly obese subjects 46.1±5.8 kg/m^2.

Plasma leptin concentrations in lean subjects and the preoperative concentrations in morbidly obese subjects ranged from 0.8-186.7 ng/ml. The leptin concentration in this population correlated strongly with BMI (r=0.796, P<0.001, Figure 6.6A). Morbidly obese subjects showed significantly higher plasma leptin levels (95.0±53.2 ng/ml) compared with lean subjects (15.5±21.3). Most interestingly, despite the large variation, sLR concentrations in obese subjects were significantly lower (21.8±47.4 ng/ml) compared with lean subjects (81.2±143.2). There was a significant inverse correlation of sLR levels with BMI (r=0.294, P<0.05, Figure 6.6B). However, leptin and sLR did not correlate with each other.

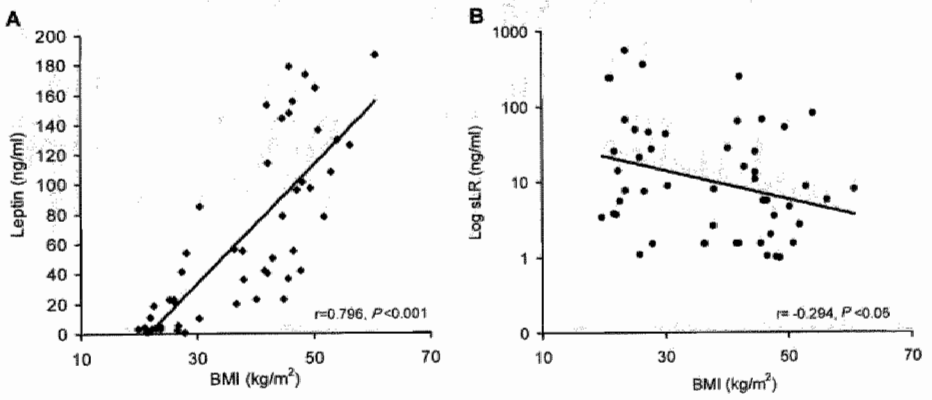

Figure 6.6 Correlation of BMI with leptin and sLR levels in a study population with a wide range of BMI. Correlation between BMI and plasma leptin levels (A) and between BMI and plasma sLR levels (B) in lean subjects and in the morbidly obese subjects before gastric restrictive surgery (n=51). The Pearson correlation coefficients are depicted in both figures.

After gastric restrictive surgery, BMI of morbidly obese subjects decreased significantly from a BMI of 46.1±5.8 before surgery to 39.6±5.5 at 3 months, 35.5±5.0 at 6 months and 33.2±5.6 kg/m^2 at 12 months postoperative. Concomitantly, leptin levels decreased dramatically from 95.0±53.2 before surgery to 44.5±28.2 ng/ml at 3 months postoperative. On the other hand, sLR levels did not change the first 3 months postoperatively (28.5±57.5 and 21.8±47.4 ng/ml, 3 months postoperative and preoperative, respectively) in these still obese subjects. In the following 9 months of weight loss, sLR levels increased significantly to 34.8±51.9 ng/ml at 6 months to reach 39.3±74.7 ng/ml at 12 months postoperative (Figure 6.7A). However, in accordance with the mean BMI of 33.2±5.6 kg/m^2 at 12 months, which is still substantially higher than the BMI of lean healthy subjects, leptin levels remain significantly elevated.

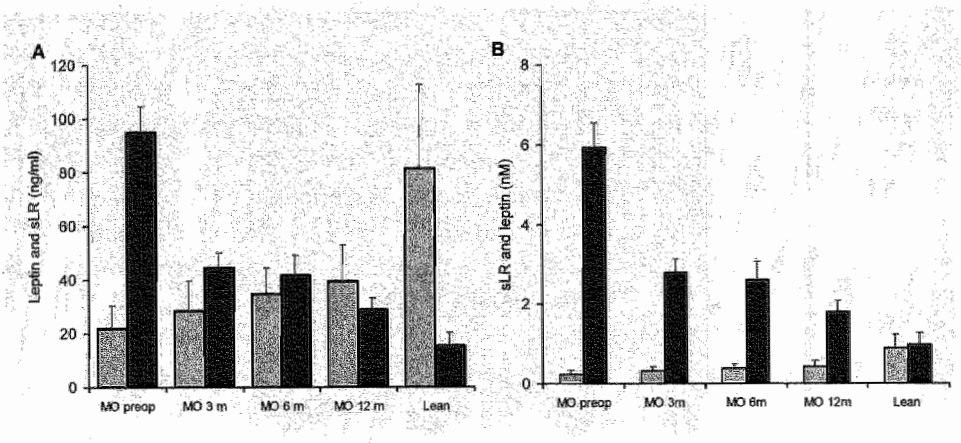

Figure 6.7 Plasma sLR and leptin concentrations in lean and morbidly obese subjects as well as in weight-losing morbidly obese individuals. Plasma concentrations of leptin (*grey bars*) and sLR (*dark grey bars*) in ng/ml (A) or in nanoMolar (B) in lean and morbidly obese subjects (MO). For the morbidly obese group preoperative (preop) as well as 3, 6, and 12 months postoperative (3m, 6m and 12m) values are shown. Data are depicted as mean ± SEM.

Discussion

In this report, we describe an investigation on the circulating soluble leptin receptor. The studies started with the generation of mAb against the extracellular part of the leptin receptor. The mAb allowed detection of sLR by Western blot and quantification by ELISA. Western blot analysis of sLR isolated from plasma with Sepharose beads with anti-sLR mAb revealed a molecular

mass of approximately 180 kDa. This is slightly higher than the reported molecular mass of approximately 140 kDa (although also molecular masses of 110 kDa and 290–300 kDa were reported[18,22–24]) for human sLR and murine sLR[25]. In our gel filtration experiments, sLR was found in fractions containing proteins with a molecular mass of 160–180 kDa. The differences in molecular mass for sLR, of which the core protein molecular mass is approximately 90 kDa, may be caused by differences in level of glycosylation as reported[18,24,25].

In plasma, sLR have been reported to circulate both in free form and bound to leptin[16,26]. Two sets of experiments supported the presence of circulating sLR-leptin complexes. First, Western blot analysis on plasma- immunoprecipitated sLR demonstrated the presence of both leptin and soluble leptin receptor. Second, incubation of plasma with Sepharose bound leptin resulted in a replacement of leptin bound to sLR by Sepharose bound leptin, leading to enhanced free leptin levels. Next, we assessed whether the developed sLR-ELISA measured apart from free sLR, also sLR complexed with leptin. To this end, plasma-derived sLR and recombinant sLR-Fc were incubated with different leptin concentrations and quantified. Formation of sLR-leptin complexes was confirmed by incubation of sLR-Fc with ^{125}I-leptin. The results demonstrated that the presence of leptin did not affect detection of sLR, indicating that both free and leptin bound sLR are measured by the sLR-ELISA. Based on these results, we had expected to detect leptin in sLR-containing fractions in gel filtration experiments in analogy to Lewandowsky et al.[22]. The latter used a RIA and demonstrated leptin in such sLR-containing fractions. However, leptin was only detected in fractions with a molecular mass of approximately 16 kDa. This prompted us to investigate whether the presence of sLR in plasma affects the quantification of leptin. In four widely used leptin assays, leptin detection was studied in the presence of plasma derived sLR or recombinant sLR-Fc. Both types of sLR inhibited the detection of leptin in a dose depended fashion. This explains that leptin was not measured in the gel filtration fractions containing sLR. Moreover, this implicates that these assays primarily measure free leptin and underestimate the total amount of leptin present in plasma because the amounts of sLR present in plasma are in the range in which strong inhibition of leptin detection was seen.

Next, we studied the free leptin and sLR concentrations in plasma of lean and morbidly obese subjects, using the above mentioned assays. In lean individuals (BMI<30 kg/m^2), a mean sLR concentration of 81.2 ng/ml was found, whereas in morbidly obese subjects (BMI>40 kg/m^2), a significantly lower mean sLR concentration of 21.8 ng/ml was observed. A significant inverse correlation of sLR with BMI was found. As expected, and consistent with previous reports[19,27], significantly higher leptin levels were observed in morbidly obese individuals compared with lean subjects. Considering the molar ratio of these proteins it appeared that in plasma of lean subjects the molar

ratio of free leptin to sLR was 1:1, with a maximal leptin to sLR ratio of 2:1 in case all sLR were saturated with leptin. In contrast, in morbidly obese subjects a ratio of 25:1 was found (Figure 6.7B). Thus, on a molar basis, we observed in plasma always more leptin than sLR. These data are supported by Sinha et al.[18], who demonstrated that in lean subjects leptin circulates mainly in the bound form, whereas in obese subjects the majority of leptin circulates in the free form.

A significant decrease of BMI, following gastric restrictive surgery, was associated with a decrease of leptin concentrations and an increase of sLR levels. However, in the first 3 months after gastric restrictive surgery, which caused a strong reduction of food intake, leptin levels dropped dramatically, whereas sLR concentrations remained unchanged. This suggests that neither food intake, nor leptin levels, were responsible for the reduced sLR-concentrations observed in morbidly obese subjects. In line with these data, Dr. C.J. Hukshorn (Department of Human Biology, University of Maastricht, personal communication) found that sc injections of recombinant leptin to moderately obese individuals, leading to very high leptin plasma levels (>5 μg/ml), did not affect sLR-levels (measured with our ELISA).

Various functions for the circulating soluble leptin receptor have been proposed. Analogous to circulating receptors of various cytokines, sLR has been reported to function as inhibitor or stabilizer of leptin[16,24]. Recombinant sLR inhibited leptin binding to COS7 cells, expressing membrane bound full-length human leptin receptor[18], which supports the inhibitory properties of sLR. In another study, an increase in plasma leptin levels was observed in rats injected with an adenovirus encoding for the sLR[28]. The latter data were explained as being proof for a stabilizing function of sLR, probably by reducing leptin clearance, implicating that sLR play a role in determining the amount of leptin in circulation. The authors of this study postulated that this may be an important mechanism to regulate the bioavailability of leptin. In line with this, Lahlou et al.[29] demonstrated high levels of leptin in patients with high sLR levels due to a defect in the leptin receptor.

Regulation of sLR could be a physiological way of regulating leptin levels in lean individuals, which have a much higher sLR/leptin ratio than obese individuals. Since leptin was reported to have strong immunoprotective effects[30,31], it could be important to enhance the bioavailability of leptin in lean individuals with low leptin levels. High free leptin levels were observed in morbidly obese subjects, probably caused by high leptin release by adipocytes due to abundant food intake. Interestingly, during food restriction, circulating leptin levels in lean and obese subjects drop dramatically within one day after onset of starvation[32], indicating that leptin release is strongly reduced. In context of the proposed stabilization function of sLR, the low sLR levels in

morbidly obese individuals, could be part of a feedback mechanism aimed at reducing the increase in leptin.
At this stage, it is unclear why exogenous leptin does lead to weight reduction in CD-1 and high fat diet induced obese mice[33,34], whereas such effects were not observed in obese individuals treated for 12 weeks with leptin-PEG[35]. An increase in the bioavailability of leptin in ob/ob mice by overexpression of sLR led to an improved weight reducing effect of exogenous leptin. In this context, it remains to be investigated whether the inability of exogenous leptin to induce body weight reduction in man is due to a lack of sLR. Plasma sLR might function like the soluble IL-6 receptor for the cytokine IL-6 (also a member of the gp-130 receptor family) and enhance leptin signalling. Hypothetically, the observed decreased sLR-levels in morbidly obese subjects might be a possibility for therapeutic interventions with recombinant sLR, instead of leptin, for the treatment of obesity.
Next to the function, the origin of sLR in plasma is also unclear. It could originate from alternative splicing of the leptin receptor or from full-length functional leptin receptors released by enzymatic cleavage. In the latter case, sLR levels in plasma could reflect the amount of leptin receptor expressed by tissues. This could implicate that decreased plasma levels of sLR as found in morbidly obese subjects are a sign of decreased expression of functional leptin receptors. This might be in agreement with the proposed leptin resistance in morbidly obese subjects[36–38]. Elucidation of such a putative role for sLR in leptin resistance may help understand the development of obesity.

In summary, we have developed methods to study human sLR and demonstrated that circulating sLR levels decrease and leptin concentrations increase with increasing body weight. Excessive weight loss following gastric restrictive surgery was shown to result in a strong decrease in circulating leptin levels and increase in soluble leptin receptor levels.

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Chapter 7

Analysis of insulin resistance and inflammatory mediators in weight losing morbidly obese subjects

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Abstract

Background
Morbid obesity is associated with elevated inflammatory mediators which are suggested to play an important role in the development of insulin resistance (IR). The aim of this study was to investigate the relationship between body weight, insulin sensitivity (measured using the steady state plasma glucose (SSPG) –test) and circulating inflammatory mediators during extensive weight loss in morbid obese patients following bariatric surgery (Group I). In addition, the influence of food intake on IR and inflammatory mediators was assessed by giving additional feeding to patients with already a stable body weight (Group II).

Methods
Group I (n=11) was measured preoperatively, at 26% and 54% excess weight loss (EWL). Group II (n=11) underwent bariatric surgery 3.4±1.8 years before enrolment in the study and reached a stable body weight with 70% EWL.

Results
Despite a significantly decrease in body mass index (BMI) in Group I to 33.7±2.5 kg/m^2 at 54% EWL, inflammatory mediators were not decreased and IR not improved. However, IR in group II (with comparable BMI but lower inflammatory mediators level) was significantly improved. Interestingly, short-term additional feeding of group II resulted in decreased insulin sensitivity without affecting inflammatory mediators and BMI.

Conclusions
Taken together, during extensive weight loss after bariatric surgery IR did not improve, possibly due to sustained enhanced levels of inflammatory mediators. After reaching a stable body weight, IR improved and inflammatory mediators decreased. However, additional feeding strongly decreased insulin sensitivity in these weight stable, obese patients.

Introduction

Obesity is an important risk factor for insulin resistance and type 2 diabetes mellitus (type 2 DM). The pathophysiological mechanism that underlies insulin resistance in obese patients is only partially unravelled. There is increasing evidence that inflammatory mediators play a central role in insulin resistance[1,2]. This was supported by data obtained with obese mice in which an important role for the pro-inflammatory cytokine TNFα was demonstrated[3]. Adipocytes and adipose tissue infiltrating monocytic cells are considered to be the source of pro-inflammatory cytokines[4-6]. Recently, we and others demonstrated that body weight significantly correlates with circulating inflammatory mediators[6,7]. However, during marked weight loss due to strongly restricted calorie intake following gastric restrictive surgery in morbidly obese patients, pro-inflammatory cytokines remained elevated up to approximately six months postoperatively[8]. Only after reaching a stable body weight, on average two years postoperatively, a substantial reduction in inflammatory mediators was observed.

In contrast to the inflammatory mediators, we found in a pilot study that fasting glucose and insulin levels immediately decreased after surgery. This does not implicate a direct decrease of insulin resistance, although as calculated according to the homeostatic model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI), insulin resistance in such patients is considered to be reduced. Others also found a direct postoperative improvement of insulin sensitivity, measured with HOMA or QUICKI[9,10].

These data led us to investigate the relation between inflammation and insulin sensitivity prior to, during and after the period of extensive weight loss following gastric restrictive surgery. The insulin sensitivity was measured with the steady state plasma glucose test (SSPG-test).

Besides inflammatory mediators, also hyperphagia has been reported as inducer of insulin resistance. Wang et al. demonstrated in an animal-study with normal rats that short-term overfeeding induced insulin resistance, measured with the hyperinsulinemic clamp technique[11]. Morbid obesity is apart from an increase in fat mass also accompanied by a relatively increased food intake which could play an important role in obesity related insulin resistance and/or inflammation. In order to study this, the effect of additional calorie intake on insulin sensitivity was studied in weight stable patients, a few years after gastric restrictive surgery.

Research design and methods

Study design

In total 22 consecutive subjects admitted to the Surgical Department of the University Hospital Maastricht for surgical treatment of morbid obesity, participated in the study. Two sub-studies were performed. In the first study, 11 morbidly obese subjects were included, 1 male and 10 females. Seven patients underwent vertical banded gastroplasty (VBG) and four subjects a Lap-Band operation. In this study the effect of extensive weight loss, caused by drastic reduction in nutritional intake after bariatric surgery, on insulin resistance and inflammatory markers was evaluated.

In the second study 11 consecutive subjects were included, 2 males and 9 females. These subjects were on average 3.4 years after their surgical treatment for morbid obesity. Nine patients underwent a VBG operation and 2 patients a Lap-Band operation, both gastric restrictive procedures. At the moment of the experiment, all subjects had reached a stable body weight for at least one year. In this study the effect of high caloric intake on insulin resistance and inflammatory markers was investigated. In both study groups, the subjects were otherwise healthy according to history, clinical examination and routine laboratory tests. In particular none of the studied subjects had evidence of diabetes mellitus or inflammatory disease or were taking medication known to influence glucose metabolism. Characteristics of both study populations are presented in Table 7.1. The study was approved by the ethical committee of the University Hospital Maastricht, the Netherlands. All subjects gave written informed consent.

Table 7.1 Characteristics of both study populations on different time points.

Variables	Preoperative Group I (n=11)	26% EWL Group I	54% EWL	Group II[a] before feeding
Sex (female / male)	9 / 2			9 / 2
Age (yr)	40.4 ± 7.6			39 ± 18.5
BMI (kg/m^2)	43.9 ± 3.5	39.4 ± 3.4	33.3 ± 2.7	31 ± 4.9
Waist/hip ratio	0.97 ± 0.08	0.93 ± 0.1	0.92 ± 0.11	0.87 ± 0.09
% EWL	-	26.3 ± 3.6	54 ± 7.1	70 ± 13.8
Weight loss (kg)	-	14.9 ± 3.3	29.8 ± 6.9	47 ± 6.7
Fasting glucose (mmol/l)	5.4 ± 0.6	5.5 ± 0.7	5.0 ± 0.7	5.5 ± 0.6
Fasting insuline (mU/l)	14.8 ± 7.8	7.4 ± 4.5[b]	6.0 ± 3.7[b]	4.5 ± 2.9
HOMA-IR	3.59 ± 1.99	2.09 ± 1.02[b]	1.48 ± 0.75[b]	1.11 ± 0.55
QUICKI	0.32 ± 0.03	0.35 ± 0.02[b]	0.37 ± 0.03[b]	0.39 ± 0.04
Days after surgery	-	53 ± 26	167 ± 78	1228 ± 394

[a] Mean BMI before surgery was 47.4 kg/m^2; [b] $P<0.05$ compared to preoperative situation.

Steady state plasma glucose test

Insulin sensitivity was determined by using the SSPG-test, described by Reaven et al.[12,13]. This insulin suppression test determines a steady state plasma glucose (SSPG) concentration as a measure for insulin mediated glucose uptake and highly correlates (r>0.90) with the golden standard, the hyperinsulinemic euglycaemic clamp technique[14]. In short, after a 12-hour overnight fast, body weight and body height were measured. Subjects were studied supine in a hospital bed. Catheters were placed in both antecubital veins to enable infusion of glucose, insulin and octreotide as well as taking of blood samples. During an acclimatisation period of thirty minutes an automatic blood pressure measurement (Dinamap, Criticon Inc., Tampa, Fl) was performed. Octreotide (Sandostatin, kindly provided by Novartis Pharma B.V. Arnhem, the Netherlands), a somatostatin analog, was administered intravenously by using a syringe infusion pump (Treonic IP4, Vickers Medical, England) at 5.0 μg/min, preceded by a bolus of 25μg. In order to optimally block insulin secretion in the morbidly obese patients, a higher infusion rate was used than described by Pei and Reaven[13]. Insulin (Actrapid, Novo-Nordisk, Bagsværd, Danmark) was infused at a rate of 25 mU/m²/min by a second syringe pump. Glucose was infused via a volumetric pump (IVAC 591, IVAC Corporation, San Diego, CA) at 240 mg/m²/min. Before the SSPG test as well as on different time points during the test blood samples were taken. The mean plasma level of glucose at 150, 160, 170 and 180 min was defined as steady state plasma glucose concentration (SSPG-concentration). SSPG-concentrations provide an indirect measure for insulin resistance.
Plasma glucose was determined by a glucose oxidase method (YSI model 2300 Stat, Yellow Springs Industries, Yellow Springs, OH). Blood samples for determining inflammatory mediators and insulin were immediately put on melting ice and plasma was prepared by centrifugation at 1,400*g* for 10 min at 4°C. The plasma was spun again at 2,700*g* for 10 min at 4°C and recovered plasma was stored in aliquots at -80°C until measurement.

Study protocols

In the first (prospective) study morbidly obese patients were studied with the SSPG test prior to gastric restrictive surgery (baseline) and after 25% and 50% excess weight loss (EWL).
In the second study (an interventional study) all subjects were studied twice. Six subjects were studied while on their normal diet after gastric restrictive surgery and subsequently after one week of additional feeding (see next page). In the other five subjects the inverse sequence was used (randomised cross-over design). To minimise the effects of the first SSPG-test on the second, the mean interval between the two tests was 18 days (range 13-24 days)

The feeding supplement consisted of three packets daily of 200 ml liquid nourishment (Resource Energy Drink by Novartis Nutrition, the Netherlands), with a total energy content of 900 kcal, with 113 gram carbohydrates and 35 gram fat per day.
In the week before the SSPG-test the subjects were requested to keep a 3-day food-intake diary (registration was performed at 1, 3 and 5 days before the test) to calculate total energy intake during additional feeding and during their habitual diet. Participants received detailed written and oral instructions on how to fill in the diaries. They were also asked to take the supplement between their usual meals in order to reduce satiety effects and to return all empty packets, in order to evaluate the amount of extra feeding consumed. The nutrient intake from food records were analysed by using international food composition tables[15].

Vertical banded gastroplasty

In our hospital the procedure was performed as initially described by Mason[16]. In short, a small pouch of the stomach (approximately 15-20 ml) was created with a 4-row linear stapler precisely to the angle of His, and a Dacron band of 5.0 cm in circumference, placed through the window formed by a circular stapler leaving a very small opening for food to pass from the small pouch to the remaining stomach. Because of the small capacity of the gastric pouch, the amount of ingested food is considerably limited, leading to extensive weight loss.

Laparoscopic gastric banding

The Lap-Band (INAMED, Carpinteria, CA) is a surgical technique used to reduce body weight. This procedure was initially performed as described by Belachew[17]. In short, the Lap-Band (9.75 cm band) was placed laparoscopically around the stomach. The band was fixed to the stomach with three or four sutures to create a small pouch of approximately 15 ml above the band. Six weeks postoperatively the Lap-Band was insufflated when weight loss was insufficient (less than 6 kg). During the study period the Lap-Band was insufflated as often as needed, up to a maximum of 4.5 ml, to induce sufficient weight loss.

Reagents, materials and assays

Monoclonal antibodies (mAbs) specifically directed against soluble human TNFα receptor 55 (TNFR55) and soluble TNFα receptor 75 (TNFR75) were obtained as described[18]. Polyclonal rabbit antisera anti-TNFR55 and anti-TNFR75 were obtained by immunising rabbits with recombinant human

TNFR55 and TNFR75, respectively. Monoclonal antibodies 4G1 and 4F8, specifically directed against leptin, were kindly provided by Dr. R. Devos (Hoffmann La-Roche, Welwyn Garden City, UK).
Human recombinant Lipopolysaccharide binding protein (LBP), used as standard, was produced by transfected Chinese Hamster Ovary (CHO) cells, kindly provided by Dr. P. Tobias (Research Institute of Scripps Clinic, La Jolla, CA). Polyclonal antibodies to human LBP were obtained by immunising rabbits with human LBP. Human C-reactive protein (CRP) was obtained from Dade Behring (Deerfield, Ill); rabbit anti-human CRP and rabbit anti-human CRP-HRP were purchased from DAKO (Glostrup, Denmark). Human alpha-1 acid glycoprotein (AGP) was obtained from Sigma (St. Louis, MO) and rabbit anti human AGP from DAKO (Glostrup, Denmark). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Recombinant human leptin was purchased from R&D systems (Minneapolis, MN). Peroxidase-conjugated streptavidin was purchased from Dakopatts (Glostrup, Denmark) and TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate from Kirkegaard & Perry Lab (Gaithersburg, MD). Immuno maxisorp plates (Nunc, Roskilde, Denmark) were used for ELISAs.
Plasma concentrations of soluble TNFα receptors, leptin, LBP, CRP, AGP and insulin concentrations were measured using sandwich ELISA's. TNFR55, TNFR75, LBP, CRP and leptin were detected as described elsewhere[6,18,19]. Plasma insulin concentrations were measured using a commercial available Elisa (Mercodia AB, Uppsala, Sweden). Free fatty acids (FFA) serum levels were measured using a commercial kit from Wako-Chemicals GmbH (Neuss, Germany).
All plasma samples were analysed in the same run, except for insulin and FFA concentrations. When plasma concentrations exceeded the upper detection limit of the assay, samples were additionally diluted and analysed in a separate run with an overlap. The intra- and inter-assay coefficients of variance of the various assays were <10%.

Statistical analysis

Data were expressed as mean ± standard deviation. The Friedman test and the Wilcoxon signed-rank test were used to analyse differences in time. Group comparisons were performed by Mann-Whitney U test. Sprearman's rho correlation coefficients were computed between the parameters under investigation. To study correlations between inflammatory mediators and SSPG-levels, data of group I preoperatively, at 26% EWL and at 54% EWL were used.

All *P*-values are two-tailed and a value of $P<0.05$ was considered statistically significant. Statistical analyses were done using the SPSS 11.0 statistical package.

BMI of both study groups

Table 7.1 summarises the characteristics of the 22 patients studied in the two studies. In the first study group mean BMI (± SD) decreased progressively from 43.9±3.6 kg/m² (preoperative) to 39.4±3.4 kg/m^2 at 26±4% excess weight loss (EWL) and subsequently to 33.3±2.7 kg/m^2 at 54±7% EWL. The 26% EWL was reached at 53±26 days and the 54% EWL at 167±78 days after surgery.
The study population of group 2 had a preoperative BMI of 47.4±6.4 kg/m². At the time of the experiment, all patients had reached a stable body weight of 89.0±18.9 kg. One year before the experiment body weight was not significantly different (91.2±23.5 kg). BMI at the time of the experiment was 31.3±4.9 kg/m^2 (70.0% EWL), which was comparable with the BMI of the subjects of the first study at 54% EWL. The mean time after gastric restrictive surgery was 3.4 years.

Effect of weight loss on metababolic parameters

As shown in Table 7.1, fasting glucose levels before surgery were in a normal range (<5.6 mmol/l). Following surgery, fasting glucose levels did not change on 26 and 54% EWL. In contrast, insulin as well as HOMA-IR decreased and QUICKI increased significantly after surgery. This led us to study the insulin resistance before and after surgery.
In Figure 7.1 the glucose levels on the different time points of the SSPG-test of both group I and II are depicted. During the experiment, glucose levels in group I increased to reach the SSPG-concentration (mean of glucose levels from t=150 to t=180) of 14.4±2.7 mmol/l. This concentration is much higher compared to the SSPG-concentrations found in healthy subjects (4.1±0.4 mmol/l), indicating a low preoperative insulin sensitivity in our morbidly obese subjects (data not shown). These data represent the lower 30th percentile of insulin resistance as measured by the group of Reaven in 490 healthy volunteers[20].
Interestingly, the mean preoperative SSPG-concentration of the subjects of the first study group was not significantly different compared to the mean SSPG-concentration after 26% EWL (14.3±5.3 mmol/l) and 54% EWL (13.4±4.6 mmol/l), respectively.
In order to evaluate whether the SSPG-tests are performed under the same conditions and data are comparable, also insulin levels were measured. As depicted in Figure 7.2, insulin levels increased during the SSPG-test, and reached a stable level at t=120. No significant differences were observed in

insulin levels at t=0, t=120 min. and 180 min. between the preoperative group and the group at 26% EWL. However at 54% EWL, insulin levels at t=0 and t=180 min. were significantly lower than observed preoperatively (P=0.003), which implicates that insulin sensitivity is probably slightly improved at 54% EWL compared to preoperative situation.

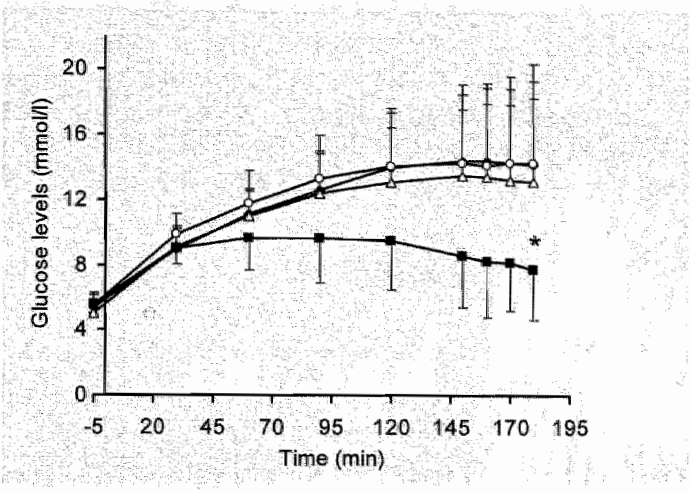

Figure 7.1 The effect of weight loss on glucose levels during the SSPG test.
Glucose concentrations during the SSPG test of 11 subjects of the first study group, before operation (black diamonds) and at 26% (white circles) and 54% (white triangles) EWL and data of the second study group with a stable body weight before feeding (black squares). Mean SSPG concentration between group I at 54% EWL was significantly lower compared to SSPG result of group II before feeding (*; P<0.01).

SSPG, weight loss and FFA levels

Various studies have suggested an influence of FFA on insulin action[21-23]. In order to study the relation of FFA and insulin resistance, plasma levels of FFA were measured in group I, preoperative, 26 and 54% EWL. As shown in Table 7.2 FFA levels did not change postoperatively on the different time points. At the same time, SSPG levels also did not change. When FFA and insulin resistance were correlated, no significant correlation was found.
In group II, with a stable body weight and a significant lower SSPG level compared to group I at all time points, a not significant decrease in FFA levels was found compared to group I at all time points. Also in this study group FFA levels did not correlate with SSPG levels.
In short, the data from both groups suggest a minor role for FFA in the pathophysiology of insulin resistance.

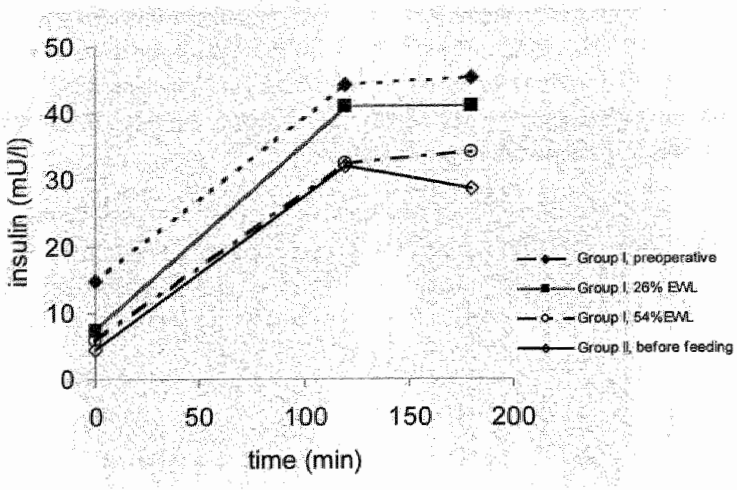

Figure 7.2 The effect of the SSPG test on insulin levels in both study groups on different time points.

Table 7.2 Levels of inflammatory mediators at t=0 during the SSPG test of both study groups.

Variables	Preoperative (n=11)	26% EWL (n=8)	54% EWL (n=11)	Group II (n=11)	Group II (after feeding)
Leptin (ng/ml)	67.0 ± 31.5	26.9 ± 14.3[a]	9.7 ± 6.2[a]	15.7 ± 16.3	18.3 ± 17.6
CRP (µg/ml)	20.3 ± 12.1	21.9 ± 17.1	8.7 ± 7.0[b]	2.3 ± 2.2	1.9 ± 1.3
AGP (ng/ml)	12.1 ± 7.7	10.3 ± 5.2	9.0 ± 4.6	10.5 ± 8.1	8.5 ± 7.9
LBP (µg/ml)	36.2 ± 16.9	35.1 ± 11.6	30.0 ± 10.7	12.7 ± 5.2	15.6 ± 4.4
soluble TNFR55 (ng/ml)	0.60 ± 0.21	0.67 ± 0.20	0.61 ± 0.11	0.72 ± 0.27	0.58 ± 0.21
soluble TNFR75 (ng/ml)	1.07 ± 0.43	1.34 ± 0.47	1.22 ± 0.33	1.26 ± 0.66	0.98 ± 0.66
FFA (mmol/l)	0.71 ± 0.22	0.80 ± 0.18	0.72 ± 0.20	0.59 ± 0.20	0.58 ± 0.20

[a] $P<0.01$ compared to preoperative; [b] $P<0.05$ compared to 26% EWL. Data is depicted as median and interquartile ranges (25%, 75%).

SSPG levels and inflammatory mediators

As expected, leptin levels were significantly decreased at 26 and 54% EWL (see Table 7.2). Besides leptin, the data show that only CRP levels were significantly decreased postoperative at 54% EWL. All other inflammatory mediators did not change, despite the extensive weight loss, on the different time points.

After reaching a stable body weight (group II), the inflammatory mediators CRP and LBP were significantly lower compared to the first (weight losing) study

group with a similar body weight (at 54% EWL (P<0.001)). AGP and both soluble TNFα receptors were not significantly different between both groups.
In order to study the relation between inflammatory mediators and insulin resistance, the SSPG levels (as an indirect measure for insulin sensitivity) were correlated with the inflammatory mediators measured. Data of group I preoperative, at 26% EWL and at 54% EWL were used. As shown in Figure 3, CRP correlated positive and significantly with SSPG levels (r=0.554, P=0.001). Moreover, when only data obtained at 26% EWL and 54% EWL were used, still a strong significant correlation between CRP and SSPG results were found (r=0.604, P=0.006). These data might implicate a relation between insulin sensitivity and inflammation, with CRP as risk indicator for the development of insulin resistance.

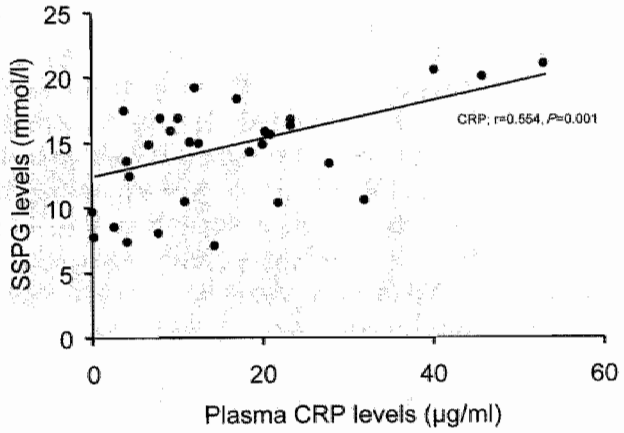

Figure 7.3 SSPG concentrations significantly correlate with CRP.

Effect of additional feeding on insulin sensitivity in weight stable patients

In group II (with a stable body weight, minimally 1.5 years after gastric restrictive surgery), the mean SSPG concentration was 8.2±3.2 mmol/l, which was significantly lower compared to SSPG concentrations of the first study group at 54% EWL (P<0.01, Figure 7.1). These date indicate that in these patients with a stable body weight insulin sensitivity is improved compared to group I with comparable body weight and to the preoperative situation of group I.
As shown in Table 7.3, the extra feeding led to a 53% increase in daily calorie intake and a 59% increase in carbohydrate intake (both P=0.005). This

additional feeding did not lead to changes in other dietary habits (food as well as liquid intake) in the period of extra food intake as assessed by food records. Furthermore, the short period of extra consumption did not lead to a change in body weight or waist hip-ratio.

Table 7.3 Anthropometrical and physiological parameters of the subjects of the second study before and after feeding[a].

Variable	Before Feeding n=11	After Feeding n=11	*P*-value
Energy intake (kcal/day)	1227.6 ± 394.4	1879.2 ± 298.4	*P*=0.005
Carbohydrate intake (grams/day)	134.0 ± 394.4	214.2 ± 32.9	*P*=0.005
Body weight (kg)	89.0 ± 18.9	89.8 ± 19.5	NS[a]
BMI (kg/m²)	31.3 ± 4.9	31.5 ± 5.1	NS
WH-ratio	0.9 ± 0.1	0.9 ± 0.1	NS
MAP[b] (mmHg)	92.5 ± 12.9	93.0 ± 9.9	NS

[a] Not significant; [b] Mean arterial pressure.

Figure 7.4 shows glucose concentrations on the different time points during the SSPG test of group II before and after feeding. The SSPG concentrations were remarkably higher after extra feeding. These data show that insulin sensitivity is strongly negatively influenced by high caloric intake in obese subjects with a stable body weight. Furthermore the data show that this effect on insulin sensitivity is rapidly lost after a short washout period. Analysis of inflammatory mediators shows that the inflammatory status was not affected by additional feeding (Table 7.2).

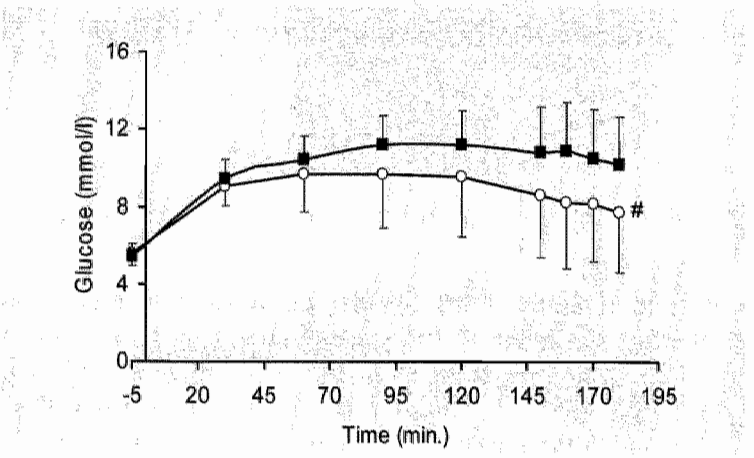

Figure 7.4 The effect of additional feeding on SSPG levels.
Accumulated SSPG data before (white circles) and after additional feeding (black squares) of the 11 subjects of the second study group. After one week of additional feeding SSPG levels increased significantly (#, $P<0.05$).

Discussion

In this report the effect of weight loss in morbidly obese patients after bariatric surgery on insulin resistance is described. Many studies report an immediate effect of weight loss on glucose and insulin levels in morbidly obese, type 2 diabetic subjects[24-26], leading to a decrease of HOMA-IR. This suggests an immediately improvement of obesity related insulin resistance after gastric restrictive surgery. In this report the SSPG test is used for measuring insulin sensitivity. This test measures the insulin-mediated glucose uptake. In line with previous studies we observed an improved HOMA-IR and QUIKI. However, here we demonstrate that during extensive weight loss, insulin resistance, measured with the SSPG-test, does not improve in the first 6 months postoperative in non-diabetic morbidly obese subjects. The reason for this discrepancy between the HOMA-IR and SSPG results is unclear. Kim et al. demonstrated that in weight stable obese subjects HOMA-IR and SSPG data significantly correlate ($r=0.6$)[27]. Our data show that during excessive weight loss, HOMA-IR and SSPG results do not correlate. This might implicate that HOMA-IR is a useful tool in determining insulin resistance in patients with a stable body weight and not during periods of metabolic stress caused by severe weight loss. Further studies are necessary to unravel this difference in assessment of insulin resistance during extensive weight loss.

An important role in the development of insulin resistance is suggested for inflammatory mediators. To study this, both insulin resistance and inflammatory mediators were measured in the patient groups. In line with our earlier report, inflammatory mediators were higher in morbidly obese subjects compared to healthy subjects[6]. Furthermore, in this study the levels of most inflammatory mediators did not change in the first 6 months postoperatively. Interestingly, in this period of weight loss, insulin resistance also did not change (as measured with the SSPG test). Moreover, besides a relation between BMI and insulin resistance, also a direct relation between insulin resistance and inflammatory mediators was found. These data suggest an important role for inflammatory mediators in the development of insulin resistance. This hypothesis is supported by the data of patients with a stable body weight (group II), in which near normal inflammatory mediator levels correlate with significantly improved insulin sensitivity.

Our findings that insulin resistance does not improve during weight loss are in contrast to the dogma that it leads to improvement of insulin resistance[24,25,28]. In contrast to our work, McLaughlin et al. demonstrated in patients with a initial BMI of 32 kg/m^2, a decrease of insulin resistance (measured with SSPG) after 3 months of calorie restriction[29], in the subjects with a mean body weight loss of 8.7 kg, being 38% EWL. In our study with patients with an initial much higher BMI of 43 kg/m^2, we obtained at aproximately 5 months postoperative a 54%

EWL that did not affect the insulin resistance. This could be due to the fact that the weight loss (=29.8 kg) of these patients was significantly higher than in the group of McLauglin. The weight loss seen in our group resembles starvation, a metabolic stress that is known to induce insulin resistance[30-32].
Others also suggested a relation between inflammation and the development of insulin resistance[33]. Fernandez et al. demonstrated that the insulin sensitivity index, determined using the frequently sampled i.v. glucose tolerance test with minimal model analysis, was strongly associated with circulating IL-6 levels[34]. Also elevated plasma TNFα levels were shown to be associated with obesity, insulin resistance, hypertriglyceridemia and glucose intolerance[35]. Furthermore, recent data indicate that adipose tissue homing macrophages are the origin of obesity associated inflammation. Studies using genome wide expression studies (micro-array analysis) on adipose tissue of murine models of obesity induced insulin resistance, as well as studies with human adipose tissue showed that the expression of inflammatory mediators observed in adipose tissue could be attributed to the presence of large number of adipose tissue homing macrophages, suggesting that these cells are the origin of obesity induced inflammation, and indirectly of insulin resistance[36,37].
With regard to the effect of weight loss on inflammatory mediators, our data are supported by Bastard et al., who showed a sustained elevation in CRP during a short period of weight loss of 3 weeks in 14 obese non-diabetic women[38]. Similarly, Heilbronn et al. reported a sustained elevation of CRP levels after 12 weeks of very-low-calorie diet in obese subjects[39].
Hypothetically, the prolonged elevation of the inflammatory markers might be caused by an enhanced metabolic stress response due to the relative starvation. This is supported by the observation that in severely malnourished anorexia nervosa patients TNFα and IL-1β are elevated in comparison to healthy controls. After cessation of starvation these inflammatory mediators levels returned to normal[40,41]. In this context, starvation induced inflammation after gastric restrictive surgery in morbidly obese patients could be an explanation for the prolonged elevation of inflammatory mediators. The presumed metabolic stress will be relieved after reaching a stable body weight, resulting in a balance between intake and consumption accompanied by decreased levels of inflammatory mediators[8].
Taken together, our results show that during extensive weight loss insulin resistance does not improve while levels of most inflammatory mediators, remained high. After stabilisation of body weight, inflammatory mediators decrease and insulin sensitivity increases.
Apart from fat mass, morbid obesity is accompanied by a high food intake, which could also underlie insulin resistance and/or inflammation. Our data show that patients with a low stable body weight and a lower daily food intake compared to before surgery have a lower insulin resistance level, as measured

with the SSPG test. However, it is unclear whether the lower BMI, the lower inflammatory mediators, the lower energy intake or a combination of these are responsible for the improved insulin sensitivity after reaching a stable body weight. To this end, we studied the influence of additional carbohydrate intake (total energy intake of 900 kcal per day) and measured insulin resistance with the SSPG technique in weight stable patients, minimally 1.5 years after gastric restrictive surgery. Insulin sensitivity decreased significantly after one week of additional feeding. The additional feeding led to a 59% increase in carbohydrate intake, without affecting fasting plasma glucose levels and circulating inflammatory mediators. In addition, no major changes in body weight were observed. These results show that the caloric intake has a strong influence on insulin sensitivity in our population of obese individuals (BMI=31 kg/m^2). These findings are supported by data of Wang et al. who demonstrated in normal rats that short-term overfeeding induced insulin resistance as measured with the hyperinsulinemic clamp technique[11]. Next to this, recent reports have demonstrated that a macronutrient intake induces oxidative stress and inflammatory responses that could underly type 2 diabetes mellitus. A glucose and fat challenge induces oxidative stress, which was demonstrated to activate, on its turn, at least 2 major proinflammatory transcription factors, NF-κB and AP-1[42-45]. Although we observed after a short period of 7 days of additional feeding an enhancement of insulin resistance, we did not find an increase in the inflammatory state. Others reported an over-feeding study for 4-6 weeks in healthy humans (caloric surplus 22.5-26.5 kcal/kg/day), which resulted in only small weight gain without induction of insulin resistance[46]. These equivocal data implicate that future studies are needed to elucidate the role of BMI, inflammation and macro-nutrient intake on insulin sensitivity in morbidly obese subjects.

Besides inflammatory mediators, FFA are also suggested to be involved in the development of insulin resistance. Elevated plasma FFA deteriorates insulin action in both healthy subjects and in type 2 diabetic patients[22,47]. However, in the present study no correlation between SSPG levels (as measure of insulin resistance) and FFA levels could be observed. Furthermore, no difference in FFA levels was found between before and after additional feeding.

Concluding, this study demonstrates that initially, following gastric restrictive surgery, when the patient is still loosing weight, insulin resistance (measured with the SSPG test) in non-diabetic morbidly obese patients does not improve. This in contrast to studies in which an improvement of insulin resistance was reported in patients with limited weight loss. The persisting insulin resistance during weight loss might be due to a sustained enhanced inflammatory state. This can be caused by metabolic stress as a result of relative starvation. In patients with a stable body weight, usually more that 2 years after bariatric surgery, insulin sensitivity significantly improved parallel to a decrease in

inflammatory mediators. In these weight stable patients short term intake of high calorie food supplements resulted in an increase of insulin resistance, suggesting a direct relation between carbohydrate intake and insulin resistance.

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Chapter 8

Discussion and summary

Discussion and summary

Vertical banded gastroplasty (VBG) as described by Mason et al.[1] was introduced in our hospital in the early eighties. Since 1996 also the lapararoscopic adjustable gastric banding (Lap-Band) as described by Belachew[2] has been used to treat morbidly obese patients. No hard evidence was available as to which treatment is to be preferred. Therefore a prospective randomized trial was performed in 100 patients, 50 Lap-Band operated patients and 50 VBG operated patients (Chapter 2). This study demonstrated that although VBG resulted in superior weight loss in the observation period of two years, it showed significantly more severe complications and the reoperation rate was significantly higher. Moreover, the Lap-Band patients showed a significantly shorter hospital stay. These findings led to the decision that in our hospital Lap-Band is the preferred gastric restrictive operation in morbidly obese subjects. Of course one needs to be aware that there are more treatment modalities for morbid obesity besides vertical banded gastroplasty and Lap-Band. However, up till now, no specific indications for different types of bariatric surgery have been defined and generally accepted. Livingston described in 2002 a study of the National Institutes of Health Consensus Panel, which reviewed the indications and types of operations. It was concluded that banded gastroplasty and gastric bypass were acceptable operations for treating seriously obese patients[3]. However, their findings did not lead to changes in surgical strategies around the world. Probably surgeons perform the type of bariatric surgery with which they are most familiar. Nevertheless, with the increasing prevalence of obesity and thus increasing numbers of operations, more specific indications for different types of bariatric surgery are needed in the near future for the treatment of morbid obese patients.

Gastric restrictive surgery leads to a reduction in comorbidities[4-7]. One of the comorbidities which are demonstrated to improve is gastro-oesophageal reflux[7,8]. On the other hand, the development or progression of motility disorders also occurs occasionally after gastric surgery[9]. In this thesis the effect of Lap-Band and VBG on gastric myoelectrical activity, an indirect measure for evaluate gastric motility, was investigated (Chapter 3). No major changes in gastric myoelectrical activity up to 3 months after both Lap-Band and VBG were observed. These data suggest that if clinical motility problems occur after bariatric surgery, these are not due to antral myoelectrical dysfunction.

In the next chapters of this thesis the inflammatory aspect of obesity was studied. An increasing number of studies have suggested an inflammatory state in morbidly obese patients as evidenced by increased plasma concentrations of cytokines and acute phase proteins without direct clinical evidence of acute or chronic inflammation in these patients[10-12]. In order to evaluate this inflammatory state, a number of studies were performed. First, the

relation between body mass index (BMI), leptin and inflammatory mediators was studied in a patient population with a wide range of BMI (Chapter 4). The data showed that leptin strongly correlated with BMI ($r=0.82$, $P<0.001$). Morbidly obese subjects had a mean plasma leptin concentration of 53 ng/ml, while lean subjects (BMI<25) showed a leptin level of 8 ng/ml. Besides leptin many inflammatory mediators were measured in order to evaluate the correlation between BMI and these markers for inflammation. The inflammatory mediators measured in this study (CRP, SAA, AGP, LBP, PAI-1, soluble TNFR55 and TNFR75) were shown to correlate significantly with BMI.
Further evaluations, in contrast, revealed that leptin did not correlate with the acute phase protein levels. Leptin was found to be correlated with both TNF-Rs and especially in a patient group with BMI≥40, suggesting that leptin is related to the enhancement of inflammatory activity in these morbidly obese subjects.
Several studies suggest a relation between inflammatory markers and the development of obesity related disorders, like cardiovascular diseases and type II diabetes mellitus[13-17]. Interestingly, it has been demonstrated that weight loss leads to reduced obesity related comorbidity[18-20]. Based on our findings that inflammatory mediators correlated with body weight, we hypothesized that weight loss in morbidly obese subjects, following gastric restrictive surgery, would result in a decrease of inflammatory mediators and acute phase proteins, which on its turn could explain the observed reduction in obesity related comorbidity, and insulin resistance in particular.
In order to study this hypothesis, inflammatory mediators were measured in morbidly obese patients, prior to and 3, 6, 12, and 24 months postoperatively (Chapter 5). Gastric restrictive surgery led to significantly decreased BMI and leptin levels. In the first six months after operation both BMI and leptin levels decreased substantially and reached a plateau at approximately 12 months. However, in contrast to these parameters, most of the acute phase protein levels measured (eg. Lipopolysaccharide binding protein (LBP), C-reactive protein (CRP) and α1-acid glycoprotein (AGP)) remained elevated for approximately 6 months. Thereafter, these inflammatory mediators declined. Two years after gastric restrictive surgery all acute phase protein levels were significantly reduced. These data suggest an ongoing inflammatory state despite extensive weight loss for at least the first 6 months postoperative. A possible explanation might be a metabolic stress response due to relative starvation during at least these first 6 months postoperatively. This seems comparable with very malnourished anorexia nervosa patients which show also enhanced inflammatory mediators compared to healthy controls[21-23]. In addition, Allende et al. reported that after re-feeding of these anorexia nervosa patients inflammatory mediators returned to normal levels[23].

The discovery of leptin in 1995 was seen as a breakthrough in obesity research. Leptin, a cytokine which is primarily expressed by adipose tissue[24], is involved in satiety regulation in mice[25,26]. Furthermore, it was demonstrated in mice that leptin controls food intake by its interaction with the leptin receptor in the brain[27,28]. As a consequence, leptin-deficient and leptin-receptor deficient mice show an obese phenotype[27,29-31]. Administration of leptin to leptin-deficient or diet induced obese mice resulted in a significant decrease of food intake[32,33]. However, human studies have, as yet, not revealed a similar role for leptin[34]. The actions of leptin are mediated by the leptin receptor of which at least four different mRNA splice variants have been identified of which one is a circulating soluble leptin receptor. Binding of leptin with the circulating soluble leptin receptor has been suggested to increase the bioavailability of leptin in plasma[35,36] whereas others suggested a decrease of binding of leptin to membrane bound leptin receptors[37]. Thus, these soluble receptors could be involved in the satiety response. In order to study this, an ELISA for the quantification of soluble leptin receptor (sLR) was developed and characterized. Next we studied circulating soluble leptin receptor and leptin levels in lean and obese subjects as well as in obese subjects during weight loss (Chapter 6). Earlier reports suggested that sLR circulates both in free form and bound to leptin[35,38], which led us to determine free and bound leptin in plasma. sLR concentrations in morbidly obese subjects were significant lower compared to lean subjects; an inverse correlation of sLR levels with BMI was observed. In line, weight loss due to gastric restrictive surgery resulted in a significantly increase of sLR levels while leptin levels, as expected, significantly decreased. In lean subjects the molar ratio of these proteins in plasma of free leptin to sLR was 1:1, with a maximal leptin to sLR ratio of 2:1, in case sLR were saturated with leptin. In contrast, in morbidly obese subjects a ratio of 25:1 was found, resulting in primarily free leptin. Up till now, no definite function for the sLR, and its relation to leptin, is known. sLR has been reported to function as inhibitor or stabilizer of leptin[37,39], the latter probably by reducing leptin clearance, implicating that sLR plays a role in determining the amount of leptin in circulation. On the other hand, sLR might function like the soluble IL-6 receptor for the cytokine Interleukin-6 and enhance leptin-signalling. Hypothetically, the observed decreased sLR-levels in morbidly obese subjects might be a possibility for therapeutic interventions with recombinant sLR, in stead of leptin, for the treatment of obesity. Elucidation the function of sLR may help understand the development of obesity related morbidity.

As mentioned above, an increasing number of studies have demonstrated a central role for inflammatory processes in the pathogenesis of the obesity related comorbidities such as cardiovascular disease and insulin resistance[13,40,41]. In chapter 5, we demonstrated a decrease of levels of

inflammatory mediators at 2 years after bariatric surgery. Hypothetically, these decreased levels of inflammatory mediators after weight loss in morbidly obese patients may be related to an improvement of comorbidities in these patients. In order to study this, the effect of extensive weight loss on insulin resistance following gastric restrictive surgery, in relation to inflammatory mediators, was evaluated (Chapter 7). We studied insulin sensitivity in morbidly obese patients using the Steady State Plasma Glucose (SSPG) test. This was measured prior to, and on different time points after gastric restrictive surgery. Furthermore, inflammatory mediators and insulin resistance were studied in a group of patients with a stable body weight several years after gastric restrictive surgery. Insulin resistance, as measured with the SSPG test, was not improved in the first 6 months after gastric restrictive surgery in non-diabetic morbidly obese subjects. Interestingly, also most inflammatory mediators did not decrease in these patients in the same time frame. In contrast, in the patients with a stable body weight, approximately three years after gastric restrictive surgery, insulin resistance was markedly improved and the level of inflammatory mediators decreased. Thus a direct relation between insulin resistance and inflammatory mediators was found, potentially supportive for a role of inflammation in the development of insulin resistance.
It is as yet unclear whether the lower BMI, the lower inflammatory mediators, the lower energy intake or a combination of these are responsible for the improved insulin sensitivity after reaching a stable body weight. In this context, the influence of additional carbohydrate intake on insulin sensitivity in weight stable patients, minimally 1.5 years after gastric restrictive surgery, was studied. Insulin sensitivity decreased significantly after one week of additional feeding, without affecting fasting plasma glucose levels and circulating inflammatory mediators. In addition, no major changes in body weight were observed. These data show that the caloric intake has a rapid influence on insulin sensitivity in (still) obese individuals with a stable body weight after gastric restrictive surgery. This indicates that gastric restrictive surgery leads to an improvement of insulin resistance due to not only reduction of inflammatory mediators but also to a decrease in calorie intake.

In conclusion, morbid obesity is a fast growing problem in the western world. The rise in prevalence of obesity is associated with an increase in the prevalence of obesity related comorbidities. Conservative treatments show disappointing results, usually of limited duration, and only leading to a slight reduction of body weight. Currently, surgical therapy is probably the only therapeutic option resulting in long-lasting and significantly weight loss in morbidly obese patients.
The results in this thesis demonstrate that surgical treatment is a valid option for morbidly obese patients. It not only leads to a significant decrease in body

weight, but also improves obesity related comorbidities. Prevention of obesity and morbid obesity is a new main health target of our society. Until this target is achieved, surgery will play an important role in the treatment of morbidly obese patients.

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Discussie en samenvatting

Discussie en samenvatting

Zeer ernstig overgewicht (body mass index (BMI)≥40 kg/m^2 (morbide obesitas)) wordt een steeds groter probleem in de westerse wereld. In Amerika hebben op dit moment al 65% van de volwassenen overgewicht (BMI≥25) en zijn 5% morbide obees. In Nederland is het probleem nog niet zo ernstig maar toch is ook hier ongeveer 1,5% van de mensen morbide obees.
Overgewicht gaat vaak gepaard met een aantal bijkomende aandoeningen (comorbiditeiten), zoals hart- en vaatziekten, suikerziekte, hoge bloeddruk etc., welke uiteindelijk kunnen leiden tot vroegtijdig overlijden van de patiënt. Gewichtsverlies is belangrijk om deze comorbiditeiten te verminderen. Patiënten proberen op allerlei manieren om dit te bereiken, zoals diëten, sporten en zelfs het slikken van verschillende (veronderstelde eetlust-remmende) medicijnen. Echter, uit meerdere studies is gebleken dat bij morbide obese patiënten een operatie de enige behandeling is met blijvend gewichtsverlies.
In ons ziekenhuis is de maagverkleiningsoperatie "verticale maagplastiek" of "vertical banded gastroplasty (VBG) volgens Mason[1]", voor het eerst toegepast in het begin van de tachtiger jaren. Sinds 1996 wordt daarnaast de laparoscopisch aanpasbare maagband (Lap-Band)[2] gebruikt als behandeling voor morbide obese patiënten. Omdat het onduidelijk is of deze operatietechniek betere resultaten geeft (met betrekking tot oa. gewichtsverlies en complicaties na de operatie) is een prospectief gerandomiseerde studie uitgevoerd met 100 patiënten: 50 patiënten met Lap-Band en 50 patiënten met VBG (Hoofdstuk 2). Deze studie toonde aan dat de VBG operatie tot meer gewichtsverlies leidde gedurende de eerste 2 jaar na de operatie. Echter, het aantal postoperatieve complicaties en het aantal re-operaties was in deze groep significant hoger in vergelijking met de Lap-Band geopereerde groep. Bovendien hadden de patiënten met een Lap-Band een significant kortere ziekenhuisopname. Deze bevindingen hebben ertoe geleid dat in ons ziekenhuis de Lap-Band de voorkeursbehandeling is bij patiënten die een maagverkleiningsoperatie moeten ondergaan.

Naast sterk gewichtsverlies leidt een maagverkleiningsoperatie tot een vermindering van verschillende comorbiditeiten[3-6]. Zo is het terugstromen van maaginhoud in de slokdarm (gastro-oesofageale reflux ziekte) één van de aandoeningen die aantoonbaar verbeterd na een maagverkleiningsoperatie[6,7]. Echter, geregeld wordt gezien dat een maagverkleiningsoperatie tot verminderde maagbewegingen leidt, die juist gastro-oesofageale reflux ziekte laat ontstaan of verergeren[8]. De electrische maagactiviteit is een indirecte maat voor de maagbewegingen en is te meten door elektroden op de buik te plaatsen. Om te bestuderen welk effect een maagverkleiningsoperatie heeft op

de maagbewegingen is er voor en na dergelijke operaties de electrische maagactiviteit gemeten, zowel bij de Lap-Band als bij de VBG (Hoofdstruk 3). Bij beide operatietechnieken werd tot 3 maanden na operatie geen belangrijke veranderingen in elektrische maagactiviteit gevonden. Dit suggereert dat eventuele maag- en slokdarmklachten, na een maagverkleiningsoperatie, niet te wijten zijn aan veranderde maagactiviteit.

Een toenemend aantal studies laat een ontstekingsbeeld zien bij patiënten met morbide obesitas. Dit beeld wordt gekenmerkt door een verhoogde concentratie van bepaalde ontstekingsstoffen (cytokinen en acute fase eiwitten) in het bloedplasma, zonder een direct klinische aanwijzing voor een acute of chronische ontsteking in deze patiënten[9-11]. Om de invloed van gewichtsverlies (na een maagverkleiningsoperatie) op deze ontstekingsstatus te onderzoeken, zijn verschillende studies uitgevoerd.
Allereerst is bij een groep van 63 mensen, met een grote spreiding in lichaamsgewicht de relatie tussen BMI, leptine (een hormoon welke voedselinname reguleert; zie verder voor uitleg) en ontstekingsmediatoren bestudeerd (Hoofdstuk 4). Ontstekingsmediatoren zijn stoffen die worden aangemaakt indien er ergens in het lichaam een ziek orgaan of een infectie aanwezig is. Deze ontstekingsmediatoren zijn dus ook een indirecte indicator voor de ernst van de ontsteking.
De resultaten van de studie toonden aan dat de plasma leptine spiegel sterk correleerde met BMI ($r=0.82$, $P<0.001$). Morbide obese patiënten hadden een gemiddelde leptine concentratie in het bloed van 53 ng/ml, terwijl normale personen (BMI<25) daarentegen een leptine spiegel hadden van 8 ng/ml. Naast leptine zijn meerdere ontstekingsmediatoren gemeten zoals C-reactive proteïne (CRP), serum amyloid A (SAA), α1-acid glycoproteïne (AGP), Lipopolysaccharide bindende proteïne (LBP), Plasminogeen activerende inhibitor-1 (PAI-1) en de oplosbare TNFα receptor 55 en 75, welke allen een significante correlatie met BMI hadden. De leptine spiegel bleek echter niet te correleren met de spiegels van verschillende acute fase eiwitten, maar wel met beide oplosbare TNFα receptoren. Dit bleek in het bijzonder bij patiënten met een BMI≥40, hetgeen suggereert dat leptine met name in de morbide obese patiënten is gerelateerd met een toegenomen ontstekingsactiviteit.
Verschillende studies suggereren een relatie tussen ontstekingsmediatoren en de ontwikkeling van comorbiditeiten die met obesitas samenhangen, zoals hart- en vaatziekten en suikerziekte[12-16]. Daarnaast is aangetoond dat gewichtsverlies tot een verminderding van deze comorbiditeiten leidt[17-19]. Aangezien wij in Hoofdstuk 4 hebben aangetoond dat ontstekingsmediatoren met lichaamsgewicht correleren, hebben wij de hypothese geformuleerd dat gewichtsverlies bij morbide obese patiënten tot een daling van de ontstekingsmediatoren zal leiden. Deze daling in ontstekingsmediatoren zou

dan vervolgens kunnen leiden tot een daling van obesitas gerelateerde comorbiditeiten.
Om deze hypothese te bestuderen zijn ontstekingsmediatoren gemeten in bloedplasma van morbide obese patiënten zowel voor, als op 3, 6, 12 en 24 maanden na een maagverkleiningsoperatie (Hoofdstuk 5). De operatie leidde tot een significante afname van zowel BMI als de leptine concentratie. In de eerste 6 maanden na de operatie daalde zowel de leptinespiegel als de BMI drastisch en bereikten deze een plateau na ongeveer 12 maanden. Echter, in tegenstelling tot deze twee parameters, bleven de spiegels van de meeste gemeten acute fase eiwitten (zoals LBP, CRP en AGP) verhoogd tot ongeveer 6 maanden na de operatie. Hierna daalden deze parameters. Twee jaar na de maagverkleiningsoperatie waren alle acute fase eiwitten significant gedaald. Deze gegevens suggereren dat de hoge ontstekingsstatus gedurende het eerste half jaar aanhield, ondanks het aanzienlijke gewichtsverlies. Een mogelijke verklaring hiervoor zou kunnen zijn dat ten gevolge van de relatieve ondervoeding er een (metabole) stress-reactie optreedt, leidend tot de aanmaak van ontstekingsmediatoren. Een vergelijkbare situatie ziet men bij patiënten met anorexia nervosa die ernstig ondervoed zijn: bij deze patiënten zijn de ontstekingsmediatoren ook sterk verhoogd[20-22]. Daarnaast blijkt dat als je bij dergelijke patiënten de ondervoeding opheft door ze te voeden, de spiegels van deze ontstekingsmediatoren normaliseren[22]. Kortom, het lijkt erop dat ondervoeding een soort stress induceert die tot verhoogde concentraties van circulerende ontstekingsmediatoren leidt.
Als na ongeveer 12 maanden na een maagverkleiningsoperatie het gewichtsverlies stabiliseert, zal de metabole stress verminderd zijn, hetgeen op zijn beurt leidt tot een vermindering van de ontstekingsmediatoren. Het ligt voor de hand dat de comorbiditeiten vervolgens verbeteren.

De ontdekking van leptine in 1995 werd gezien als een doorbraak in het obesitas onderzoek. Leptine wordt voornamelijk geproduceerd in het vetweefsel en is als zodanig een indirecte maat voor de hoeveelheid vetweefsel[23]. Het is in muizen aangetoond dat het betrokken is bij de regulatie van de voedingsverzadiging[24,25]. Bovendien reguleert leptine de voedselinname door interactie met de leptine receptor in de hersenen[26,27]. Onder experimentele omstandigheden zullen muizen die geen leptine of leptine receptoren kunnen maken, dik worden[26,28-30]. Omgekeerd, als leptine aan muizen wordt gegeven die geen leptine kunnen maken of aan muizen die door overvoeding dik geworden zijn, dan verminderd de voedselinname sterk[31,32]. Bij de mens is dit echter niet het geval[33].
De werking van leptine is gemedieerd door de leptine receptor, waarvan minstens vier verschillende varianten zijn geïdentificeerd. Één van deze varianten is de circulerende oplosbare (soluble) leptine receptor (sLR). Er is

gesuggereerd dat binding van leptine aan de sLR leidt tot een toename van de biologische beschikbaarheid van leptine in het bloed[33,34]. Echter, anderen rapporteerden een afname in interactie van leptine met membraangebonden leptinereceptoren[35]. De sLR zouden dus een rol kunnen spelen in de regulatie van de activiteit van leptine. Om dit te bestuderen, werden sLR spiegels onderzocht. Om deze spiegels te kunnen meten werd een meetmethode voor de sLR ontwikkeld (ELISA). Vervolgens werden sLR en leptine spiegels bepaald in normale en obese personen alsook bij morbide obese patiënten tijdens gewichtsverlies (Hoofdstuk 6). Aangezien eerdere studies suggereerden dat sLR zowel vrij als gebonden aan leptine circuleren[33,36], zijn beide in het bloed bepaald.
Plasma sLR concentrations in morbide obese patiënten bleken significant lager te zijn dan in normale individuen. In overeenstemming hiermee leidde gewichtsverlies, ten gevolge van een maagverkleiningsoperatie, tot een significante verhoging van sLR en, zoals verwacht, tot een significante daling van de leptine concentraties. Bij normale proefpersonen was de molaire verhouding in het bloed van vrije leptine en sLR, 1:1 en maximaal 2:1 indien sLR volledig verzadigd zou zijn met leptine. Echter, bij morbide obese patiënten werd een verhouding van 25:1 gevonden, wat inhoudt dat in de circulatie hoofdzakelijk vrij leptine voorkomt, terwijl bij patiënten met een lage BMI voornamelijk gebonden leptine in het plasma circuleert.
Tot op heden is de functie van sLR, en zijn relatie met leptine, nog onduidelijk. Zoals hierboven vermeld is zowel beschreven dat sLR als remmer en als stabilisator van leptine zou functioneren[35,37]. Het laatste zal mogelijk veroorzaakt worden door een vermindering van de klaring van leptine, hetgeen impliceert dat sLR een rol speelt in de regulatie van de hoeveelheid vrij leptine in de bloedsomloop. Anderzijds zou sLR de leptine signalering kunnen verbeteren, zoals beschreven voor de verwante oplosbare IL-6 receptor voor de cytokine IL-6.
Hypothetisch zouden de waargenomen verminderde sLR-concentraties bij morbide obese patiënten de mogelijkheid bieden voor therapeutische interventies met recombinant sLR (in plaats van leptine) voor de behandeling van obesitas en obesitas gerelateerde comorbiditeit indien leptine invloed heeft op verzadiging. Opheldering van de functie van sLR zou kunnen helpen bij het begrijpen van het ontstaan van obesitas.

Zoals reeds eerder vermeld laat een toenemend aantal studies een centrale rol voor ontstekingsprocessen zien in het ontstaan van obesitas gerelateerde aandoeningen zoals hart- en vaatzieken en suikerziekte[12,38,39]. In Hoofdstuk 5 was aangetoond dat de plasma spiegels van ontstekingsmediatoren uiteindelijk afnemen na een maagverkleiningsoperatie. Dit kan betekenen dat de afname

van de spiegels van ontstekingsmediatoren samengaat met een verbetering van obesitas gerelateerde aandoeningen.
Om dit te bestuderen, werd het effect van gewichtsverlies na een maagverkleiningsoperatie op insuline resistentie onderzocht en gecorreleerd met de plasma concentraties van de ontstekingsmediatoren (Hoofdstuk 7). Om insuline resistentie te bestuderen in morbide obese patiënten is gebruik gemaakt van de Steady State Plasma Glucose (SSPG) test. Deze test werd op verschillende tijdstippen zowel voor als na een maagverkleiningsoperatie uitgevoerd. Daarnaast werd de SSPG test uitgevoerd in een patiëntengroep met een stabiel lichaamsgewicht, bereikt enkele jaren na een maag-vekleiningsoperatie. Ook in deze groep werd de relatie tussen de gemeten insuline resistentie en ontstekingsmediatoren bestudeerd.
Het bleek dat de insuline resistentie 6 maanden na een maag-verkleiningsoperatie, niet was verbeterd. Bovendien bleken ook de spiegels van bijna alle ontstekingsmediatoren niet afgenomen te zijn. Echter, in de patiëntengroep met een stabiel lichaamsgewicht, ongeveer 3 jaar na een maagverkleiningsoperatie, bleken zowel de insuline resistentie alsook de plasma spiegels van de ontstekingsmediatoren sterk te zijn verbeterd. Dit suggereert een relatie tussen insuline resistentie en ontstekingsmediatoren, hetgeen de hypothese ondersteunt dat ontsteking een belangrijke rol speelt in de ontwikkeling van insuline resistentie.
Het is tot op heden onduidelijk wat verantwoordelijk is voor een verbetering van de insuline resistentie na het bereiken van een stabiel lichaamsgewicht: een lagere BMI, lagere spiegels van ontstekingsmediatoren, lagere energieopname of een combinatie van deze factoren. Daarom werd de invloed van extra koolhydraat-inname bestudeerd op de insuline resistentie van patiënten, minimaal 1,5 jaar na een maagverkleiningsoperatie en met een stabiel lichaamsgewicht. Het bleek dat de insuline resistentie beduidend verslechterde na één week hyperalimentatie, zonder verandering van de nuchtere glucosewaarde en de concentraties circulerende ontstekingsmediatoren. Bovendien veranderde het lichaamsgewicht nauwelijks. Deze gegevens tonen aan dat een intake van koolhydraten een snelle invloed heeft op de insuline resistentie in deze patiënten. Dit betekent dat de verbetering van insuline resistentie na een maagverkleiningsoperatie niet alleen valt toe te schrijven aan een daling van de spiegels van ontstekingsmediatoren, maar ook aan een daling van koolhydraat-intake.

Samenvattend, morbide obesitas is wereldwijd een snelgroeiend gezondheidsprobleem. De toenemende prevalentie van obesitas gaat gepaard met een verhoging van de prevalentie van obesitas gerelateerde aandoeningen. Conservatieve behandeling is meestal niet effectief, meestal van beperkte duur en leidt slechts tot een beperkte vermindering van het

lichaamsgewicht. De chirurgische therapie is momenteel de enige therapeutische optie die leidt tot een langdurig en aanzienlijk gewichtsverlies bij morbide obese patiënten.
De resultaten in dit proefschrift tonen verder aan dat een maagverkleiningsoperatie bij morbide obese patiënten niet alleen tot een afname van lichaamsgewicht leidt, maar ook tot een afname van comorbiditeiten.
De preventie van obesitas en morbide obesitas is een nieuw belangrijk gezondheidsdoel voor onze maatschappij. Totdat dit doel bereikt is, zal de chirurgie een belangrijke rol in de behandeling van morbide obese patiënten spelen.

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Prof. dr. J.W.M. Greve, beste Jan-Willem, begonnen als mijn co-promotor ben je, na een welverdiend verworven hoogleraarschap, mijn promotor geworden. Altijd was je bereid tot overleg, zelfs tijdens je vakantie. Jouw enorme ervaring op het gebied van de bariatrische chirurgie is van onmisbare waarde geweest tijdens het onderzoek, zowel qua uitvoering als inhoudelijk. Je bent niet alleen ambitieus maar ook kritisch, zowel voor anderen als voor jezelf. Deze eigenschappen hebben gemaakt wat je nu bent: hoogleraar, opleider en president van de International Federation for the Surgery of Obesity. Hierop mag je terecht trots zijn. Onderzoek doen hield voor jou ook in dat de bevindingen moesten worden uitgedragen. Deze voordrachten brachten mij (vervelend genoeg) op menig exotische locatie (en wel van Sao Paulo tot Dakar). Behalve voor de wetenschap heb je me ook op klinisch gebied weten te enthousiasmeren, en wel voor de bariatrische chirurgie. Waarschijnlijk gaan we elkaar nog vaak tegenkomen op eerder genoemde exotische locaties en dan als collega's. Jan Willem, bedankt.

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fantastisch nieuw protocol in. Dat onze samenwerking niet is gestopt toen ik voor de heelkunde onderzoek ben gaan doen blijkt wel uit hoofdstuk 7, dat een gezamenlijk product is. Ik ben vereerd dat jij lid bent van mijn beoordelingscommissie.

De andere leden van de beoordelingscommissie Prof. dr. C.D.A. Stehouwer (voorzitter), Prof. dr. M.H. Hofker en Prof. dr. P.B. Soeters wil ik bedanken voor het kritisch doornemen van mijn proefschrift. I want to thank Prof. dr. N. Scopinaro for his critical evaluation of my thesis.

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List of publications

List of publications

Papers presented in this thesis

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Curriculum vitae

Curriculum vitae

François van Dielen werd op 7 april 1972 geboren in 's-Hertogenbosch. In 1990 behaalde hij het VWO diploma op het Jeroen Bosch College te 's-Hertogenbosch. Datzelfde jaar werd gestart met de studie geneeskunde aan de Rijksuniversiteit Limburg te Maastricht. Tijdens zijn studie was hij student-assistent op de afdelingen Interne Geneeskunde (Dr. N.C. Schaper) en Chirurgie (Dr. H.A.J.M. Kurvers). In deze periode ontving hij een "Young Investigator Award". Op 5 januari 1998 behaalde hij zijn artsexamen. Meteen aansluitend is hij bij de vakgroep Chirurgie met zijn promotieonderzoek begonnen, onder begeleiding van Prof. dr. J.W.M. Greve en Prof. dr. W.A. Buurman. Voor dit onderzoek verwierf hij medio 1999 een AGIKO-stipendium. Tijdens zijn promotieonderzoek ontving hij de "Schering-Plough prijs" en de "Pélerin-prijs". Sedert 1 juli 2002 volgt hij de opleiding tot algemeen chirurg in het Academisch Ziekenhuis Maastricht (opleider Prof. dr. J.W.M. Greve). Vanaf 1 januari 2006 heeft hij zijn opleiding voortgezet in het Catharina-ziekenhuis Eindhoven (opleider Dr. G. Nieuwenhuizen).
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